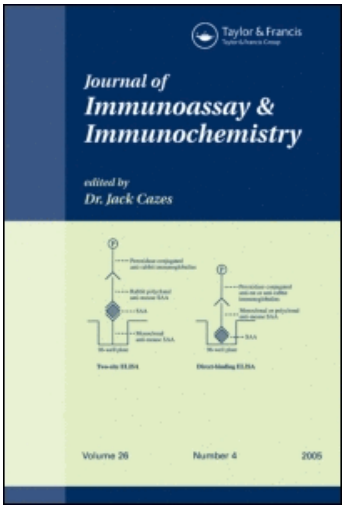

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DETERMINATION OF TRANSFORMING GROWTH FACTOR-β2 (TGF-β2) IN BOVINE COLOSTRUM SAMPLES

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ABSTRACT

Transforming growth factor-β2 (TGF-β2) is the major TGF-β form in bovine colostrum. A colostrum pool of the five first milkings was made to validate an ELISA specific for human TGF-β2 for measure TGF-β2 concentration in bovine colostrum samples. According to this test >90% of total TGF-β2 (74.5±4.4 ng/ml) in colostrum pool was in a latent form that could be activated by acetic acid treatment, whereas the concentration of the active form was only 4.19±0.27 ng/ml. Activated colostrum samples of the first milkings of five cows contained 150-1150 ng TGF-β2/ml and its concentration declined in correlation (r=0.86) with total protein concentration to 12-71 ng/ml by the fifth milkings. Most of the TGF-β2 (94%) was found in the whey fraction of colostrum. The ELISA results were also compared with a TGF-β2 bioassay, the fibroblasts migration assay. This assay detected 9.8±1.0 ng/ml and 4.4±0.7 ng/ml in the activated and non-activated samples of colostrum pool respectively.

(KEYWORDS: TGF-β, colostrum, ELISA, growth factor)

INTRODUCTION

Bovine milk and colostrum contain growth factors such as insulin-like growth factors (IGF-1 and IGF-2)(1), transforming growth factor-β (TGF-β1 and TGF-β2) (2-4) and some less defined growth factors or related molecules (5-9). Colostrum is an especially rich source of growth factors, suggesting that these growth factors may have an important role in the growth and development of calves.

In mammals the TGF-β superfamily of growth factors comprises TGF-β1, TGF-β2 and TGF-β3 (not detected in bovine milk or colostrum), bone morphogenic factors, activins, inhibins and Mullerian inhibitor (10-13). It has been proposed that TGF-β may be a mediator of mucosal immunity in milk (2), involved in the development of gut (14) and regulation of mammary gland (15). TGF-β2 gene, like other TGF-βs, encodes a large precursor molecule which is cleaved to an inactive precursor polypeptide chain. This polypeptide contains a large N terminal domain and a C terminal domain, which is the mature form of TGF-β2 (16). After proteolytic processing the N terminal domain remains non-covalently bound to the C terminal domain and this complex is known as an inactive latent TGF-β2 (17). Most forms of TGF-β are homodimers containing two identical polypeptide chains, although heterodimers such as TGF-β1,2 and TGF-β2,3 have also been found (18). Activation of the latent form by removal of the N terminal domain is induced by changes in ionic strength, acidification or proteolytic enzymes (19).

The different TGF-β assays are reviewed by Meager (19), and include bioassays such as a colony formation assay with NRK47F rat kidney cells (20), cell growth inhibition assays (21) and a fibroblast migration assay (22). In addition, specific enzyme-linked immunoassays (ELISA) have been developed for TGF-β1 (23) and TGF-β2 (24) during the recent years including the assay validated in this paper.

Tokuyama and Tokuyama (25) detected a higher level of TGF-β activity in bovine colostrum than in normal milk, although no exact quantitative measurements were presented. Rogers et al. (26) used a growth inhibition assay and detected 4.3±0.8 ng and 3.7±0.7 ng of total TGF-β/ml in bovine milk and cheese whey respectively. According to purification yields TGF-β2 is most probably the predominant TGF-β

form (85-95%) in bovine milk (3) and colostrum (4), the remainder being TGF-β1. The whole amino acid sequence of the bovine TGF-β2 and the N-terminal end (29 residues) of the bovine TGF-β1 are identical with those of their corresponding human counterparts (3).

Milk and colostrum are complex biological fluids, and this makes the quantitative analysis of specific growth factors, such as the different forms of TGF-β, very difficult. This paper focuses on the procedure for measuring the TGF-β2 content of bovine colostrum (and colostrum whey) with a TGF-β2 specific ELISA. The results were also compared with those obtained by the fibroblast migration assay.

MATERIALS AND METHODS

Collection of bovine colostrum and preparation of whey

Milk samples from the five first milkings after calving were collected from Ayshire cows, immediately frozen and stored at -20°C. Before analysis the milk samples were centrifuged at 10,000 g for 10 min (Sorvall RC-5B, DuPont, Wilmington, DE, USA) to remove lipid. The five first milkings were pooled and used to validate the ELISA kit. Samples of the pool was stored at -20°C and each sample was thawed only once before use.

Whey was prepared by a standard acid precipitation of casein from the colostrum pool. Briefly, casein was precipitated by adding sufficient 2 M HCl to defatted colostrum to adjust the pH to 4.6. The pH of the cleared whey was then adjusted to 7.0 with 4 M NaOH. Whey as divided into portions and stored at -20°C.

TGF-β2 ELISA and TGF-β standards

TGF-β2 ELISA was from R&D Systems Inc. (Abingdon OX 14 3YS, UK) and has been designed for measuring human TGF-β from serum and cell

culture samples. The ELISA measurements were carried out according to the manufactures' instructions using RD5B sample diluent buffer of the kit.

Natural bovine TGF-β2 (NIBSC/WHO interim reference standard 89/518) and natural bovine TGF-β1 (NIBSC/WHO interim reference standard 89/516) standards were from NIBSC (National Institute for Biological Standards and Control, Potters Bar EN6 3QH, UK). The TGF-β2 and TGF-β1 standards contained 5 000 U/ml (approximately 250 ng) and 1 500 U/ml (approximately 80 ng) respectively, according to NIBSC. The TGF-β2 standard of the ELISA kit contains recombinant human TGF-β2.

Fibroblast migration assay

The fibroblast migration assay was performed according to Bürk (22). It measures the number of fibroblasts that migrate from the monolayer in wounded cell culture maintained in serum free medium for 22 h in the presence or absence of test substances.

Balb/c 3T3 mouse fibroblasts (ATCC CCL-163) were seeded in Dulbeccos's modified Eagle's medium (DMEM) (Flow Laboratories Ltd, Rickmansworth, UK) supplemented with glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 8% bovine serum (Sigma Chemical Co., St Luis, MO, USA) into 24 well cell culture plates (Costar, Cambridge, MA, USA) at a concentration of 150 000 cells/ml. After 4 d incubation a wound was made in the confluent monolayer by pressing a sterile scalpel blade on to the bottom of the well to cut the layer. The wounded cell cultures were washed twice with serum-free DMEM to remove the detached cells, other debris and serum. Test samples were sterile filtered through 0.22 µm filters and diluted in 1 ml serum-free DMEM. The samples were added to washed cell cultures and incubated at 37°C for 22 h. After incubation the cells were fixed and

stained at +4°C with toluidine blue in 70% methanol. The number of migrating cells in three replicate optical fields of vision (1.9 mm) were counted using a light microscope at 100-fold magnification.

Protein assay

The total protein content of the colostrum samples was analysed according to Lowry *et al.* (27) using bovine serum albumin (Sigma) as a standard.

RESULTS

Activation of TGF-β2

TGF-β2 usually exists in the form of a latent complex in biological fluids that cannot be detected by the ELISA kit, according to the manufacturer. To measure the total amount of TGF-β2 the samples (colostrum and colostrum whey) were first activated by adding glacial acetic acid to a final concentration of 1 M and incubated at room temperature for 1 h. Dilution of the samples with the sample dilution buffer RD5B of the ELISA kit was enough to neutralize acetic acid before analysis.

The other neutralization methods (dialysis or drying in a vacuum sentrifuge) suggested by the kit manufacturer, failed. When the activated samples were dried in a vacuum centrifuge to remove acid, a lot of material remained insoluble after resolubilization of the samples in the sample dilution buffer. Alternatively, the samples were dialyzed instead of drying, but this also resulted in precipitation of some milk components. A very low level of TGF-β2 was detected in the liquid fraction after these two alternative treatments suggesting that a major portion of the growth factor might be remained associated with the precipitated material.

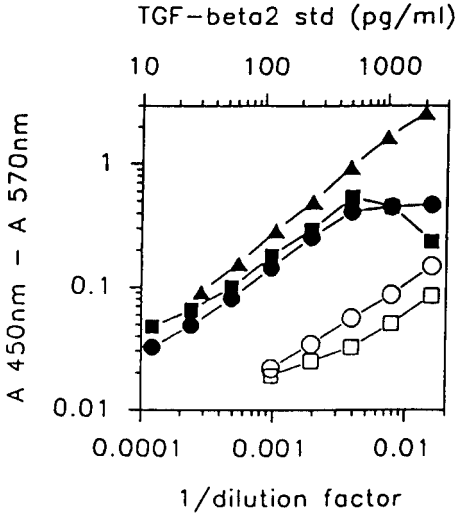

FIGURE 1 Dose-response curves for ■, activated colostrum; ●, activated whey; ❑, non-activated colostrum; ○, non-activated whey and ▲ TGF-β2 standard of the ELISA kit. The samples were diluted in RD5B buffer and the TGF-β2 ELISA was carried out according to the manufacturer. Each point represents the mean of duplicate measurements.

TGF-β2 ELISA

Linearity

Colostrum and whey pools were used to validate and optimize the ELISA. To determine the linearity of the ELISA and the minimum dilution of the activated samples dilution series of activated and non-activated samples of colostrum and whey were assayed. The results (Fig. 1) indicated that the minimum dilution of the activated colostrum and whey samples was 1:256. The non-activated samples gave significantly lower absorbances, indicating that a major portion of the total TGF-β2 was, as expected, in the latent form in colostrum and whey. The dose-response curves of the activated colostrum and whey

samples were parallel to the standard curve of the kit at dilutions 1:256-1:2048. This indicates that acetic acid and/or some other factors in the sample matrices did not interfere at high dilutions. The non-activated samples gave also parallel dose-response curves at dilutions 1:64-1:256.

Recovery

Two experiments were conducted to study the recovery of TGF-β2.Firstly, activated samples of colostrum and colostrum whey pools (dilutions 1:1000) were spiked with four concentrations (3.9-250 pg/ml sample) of TGF-β2 (the standard of the kit) and, secondly, four dilutions (1:128-1:1024) of activated colostrum and colostrum whey were spiked with 250 pg of TGF-β2/ml sample. The spiked samples were assayed by ELISA and the recoveries were calculated. The results (Tables 1 and 2) are in agreement with Fig. 1, indicating that the minimum dilution of the activated colostrum and whey samples should be >1:256. At dilutions 1:256-1:1024 the recovery of TGF-β2 was 78-107%, which corresponds to the recoveries (70-113%) obtained in different sample matrices according to the manufacture of the ELISA kit. The second experiment was also repeated with non-activated samples. The recoveries were 76-81% and 87-108% in colostrum and colostrum whey respectively.

Specificity and precision

The standard of the ELISA kit contains recombinant human TGF-β2. However, the amino acid sequences of the active forms of human and bovine TGF-β2 are identical (3) and the dose-response curves of the standard of the kit and natural bovine TGF-β2 standard (NIBSC) are parallel according to the manufacturer of the kit. Fig 1 also indicates that colostrum samples give dose-response curves that are

TABLE 1

The recovery of TGF-β2 spiked to four different levels (3.9-250 pg/ml) in activated colostrum and colostrum whey (diluted 1:1000).

TGF-β2 added to colostrum (pg/ml)	Recovery %	TGF-β2 added to colostrum (pg/ml)	Recovery %
3.91	104	3.91	97
15.6	102	15.6	101
62.5	92	62.5	93
250	93	250	107

TABLE 2

The recovery of spiked TGF-β2 (250 pg/ml) in four different dilutions of activated colostrum and colostrum whey.

Colostrum dilution	Recovery %	Whey dilution	Recovery %
1:128	31	1:128	37
1:256	78	1:256	72
1:512	87	1:512	93
1:1024	90	1:1024	104

parallel with the standard of the kit. This makes it possible to use the standard of the kit in the assays. Another possibility would have been to use the natural bovine TGF-b2 standard, although it contains a standardized amount of TGF-β activity (U/ml).

According to the manufacturer the assay does not recognize porcine and human TGF-β1 (<0.1% cross reactivity). When bovine TGF-β1 (NIBSC standard 89/518) was assayed, no cross reactivity was observed even at a concentration of 2000 pg TGF-β1/ml (result not shown), indicating that the assay is specific for bovine TGF-β2. The intra- and inter-assay CV for colostrum samples were 10.4% and 8.6% respectively.

TABLE 3

TGF-β2 concentration of the activated samples from the five first milkings of five cows. Values are ng/ml (means±SE) from triplicate measurements.

Cow	milking				
	1	2	3	4	5
1	805	220	109	65.1	62.6
2	150	156	102	72.7	71.3
3	1150	325	137	56.3	47.0
4	289	105	18.5	11.3	12.6
5	398	133	57.9	55.2	45.7

TGF-β2 content of colostrum, colostrum whey and milk

Total TGF-β2 content of the five first milkings of five Ayshire cows were analysed using the ELISA kit. The samples were defatted, activated by adding acetic acid and diluted at least 1:400 after activation. The results are shown in Table 3. The first milkings contained 150-1150 ng TGF-β2/ml and the concentration declined to 12.6-71.3 ng/ml by the fifth milkings. TGF-β2 concentration of the colostrum samples were correlated (r=0.86) with the total protein concentration (Fig.2). A sample of normal pasteurized milk (activated) from a local dairy was also analysed and contained 37.7 ng/ml.

The concentrations of the total and active TGF-β2 form in the colostrum pool were 74.5 and 4.19 ng/ml respectively, and activated and non-activated whey samples prepared from the colostrum pool contained 70.2 and 4.61 ng/ml respectively. This suggests that a major portion (94%) of TGF-β2 existed in the whey fraction of colostrum and casein precipitation at pH 4.6 activated only a minor fraction of the latent TGF-β2.

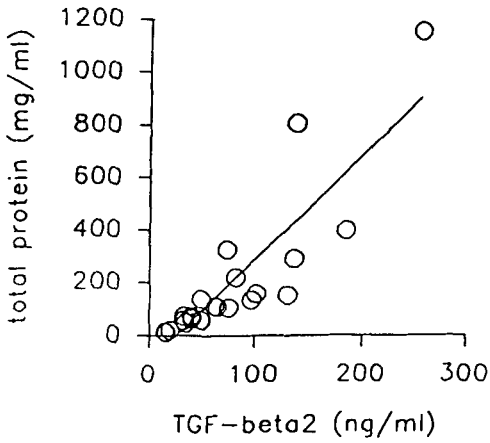

FIGURE 2 Correlation between the total protein and TGF-β2 concentration of the colostrum samples from the five first milkings of five cows. The colostrum samples were activated by acetic acid before TGF-β2 analysis. The TGF-β2 ELISA was carried out according to the manufacturer. Each point represents the mean of triplicate and duplicate measurements of TGF-β2 and total protein respectively. The correlation ($r=0.86$) was calculated using the least squares method.

Fibroblast migration assay

To compare the ELISA method and the fibroblasts migration assay an activated and non-activated sample of the colostrum pool was analysed using the fibroblasts migration assay (22). This bioassay is not specific for different forms of TGF-β, since both TGF-β1 and TGF-β2 stimulate the migration of the fibroblasts (3). However, TGF-β2 is most probably the predominant form in bovine milk and colostrum. According to Tokuyama and Tokuyama (4) TGF-β1 represents only $\leq$ 5% of the total TGF-β in colostrum and Jin et al (3) detected about 15% TGF-β1 in their TGF-β fractions purified from milk. Therefore the standard curve for this assay was constructed using TGF-β2 (NIBSC standard 89/518) and it was linear in the range 0.125-2.5 U/ml (Fig.3). For

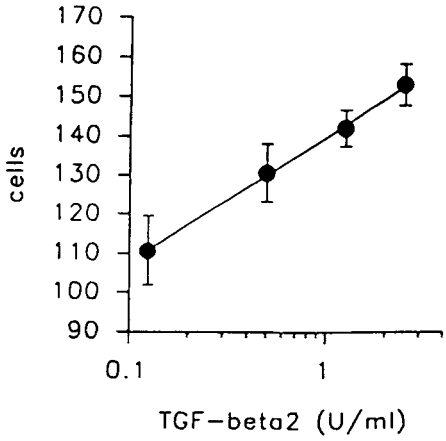

FIGURE 3 Standard curve for the fibroblasts migration assay. The line represents a linear regression of the points. Each point represent the mean±SE of triplicate measurements.

some unknown reason the TGF-β2 standard of the ELISA kit was toxic to the cells even after dialysis.

According to this assay there was 700±70 U TGF-β/ml in an activated sample of the colostrum pool, whereas a non-activated sample contained 310±50 U TGF-β/ml. To compare these results with those obtained by ELISA the NIBSC TGF-β2 standard was also assayed by the ELISA kit to determine its specific activity. According to the specific activity of the standard (71.1 U/ng) the activated and non-activated samples of the colostrum pool contained 9.8±1.0 ng/ml (74.5±4.4 ng/ml) and 4.4±0.7 ng/ml (4.19±0.27 ng/ml), respectively (corresponding ELISA results in parentheses): Thus, the ELISA kit and the bioassay gave quite similar TGF-β concentrations in the non-activated colostrum sample, but the ELISA kit detected about 8-fold higher TGF-β concentration in the activated sample.

DISCUSSION

TGF-β is a very interesting and potent growth factor that is known to cause numerous effects (11,12). Its role in milk is not known, but it has been proposed to be a mediator of mucosal immunity (2). Since TGF-β induces terminal differentiation of intestinal epithelial cells *in vitro* (14), this growth factor may also be involved in the development of gut of newborn animals. It is not known whether TGF-β is activated in milk in gut, but acidic secretions and/or proteolytic enzymes may provide a suitable environment.

TGF-β1 is the major form in serum and most tissues including spleen, liver and kidney, whereas TGF-β2 is most abundant in placenta, male submaxillary gland and lung (28,29). The predominant TGF-β form in TGF-β fractions of bovine milk (3) and colostrum (4) is TGF-β2 (85-95%), whereas the rest is composed of TGF-β1. However, no quantitative measurements of TGF-β2 concentration of colostrum were presented. The bioassays, which have usually been used to determine TGF-β, rely essentially on the ability of TGF-β to induce proliferation or migration of cells such as fibroblasts in culture. It is difficult to optimize and standardize these assays and they cannot usually measure the different forms of TGF-β in biological samples. It is also known that several other factors, such as platelet-derived growth factor, interfere in these assays (19). Immunoassays, such as ELISA, are more specific, reliable and generally reasonably sensitive to measure growth factors. In addition they are simple and rapid. Although it has proved very difficult to raise specific monoclonal antibodies against different forms of TGF-β owing to molecular conservation, some TGF-β subtype specific ELISA have been developed during recent years (23-24).

The ELISA kit used in this study has been designed for determination of human TGF-β2 concentrations in cell culture supernate, serum and plasma. Therefore, several experiments were carried out to determine the linearity and specifity of the assay for measuring TGF-β2 in bovine colostrum (and whey) samples, which are very complex sample matrices like serum. The results indicated that the ELISA was suitable for measuring bovine colostrum samples when the activated samples were diluted at least 1:256.

NIBSC provides a natural bovine interim reference standards for TGF-β1 and TGF-b2. This makes it possible to compare different TGF-β2 analysis methods. These standards contain a standardized amount of TGF-β activity, although their growth factor content in weight units is not measured exactly. The TGF-β2 content (in ng/ml) of the NIBSC standard was measured by the ELISA in order to compare the results obtained by the fibroblast migration assay and ELISA. The ELISA kit detected 74.5 ng TGF-β2/ml in an activated sample of colostrum pool, whereas the fibroblast migration assay gave only about 10 ng/ml. On the other hand, the fibroblast migration assay and the ELISA gave quite similar results when non-activated samples of the colostrum pool was analysed (about 4 and 4.19 ng/ml, according to the fibroblast migration assay and ELISA respectively). These results suggest that the activity of TGF-β in colostrum and milk could also be regulated by some mechanism(s) other than activation of the latent complex, alone.

The highly specific immunoassays, such the TGF-β2 ELISA descibed in this study, provide the possibility to investigate the changes in TGF-β2 concentration in colostrum and in more detail.

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