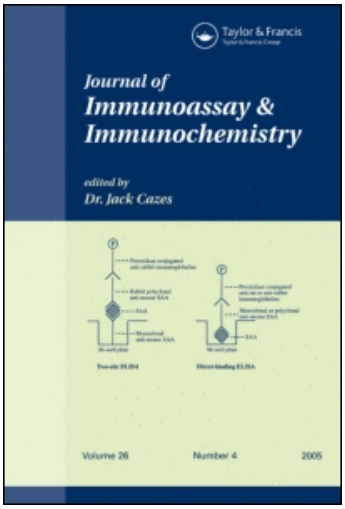

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DETERMINATION OF TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) AND INSULIN-LIKE GROWTH FACTOR 1 (IGF-1) IN BOVINE COLOSTRUM SAMPLES

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ABSTRACT

The major growth factors in bovine colostrum are transforming growth factor-βs (TGF-β1 and TGF-β2) and insulin-like growth factors (IGF-1 and IGF-2). Recently, TGF-β2 content of bovine colostrum was measured using a TGF-β2 specific ELISA (1) and now we have validated ELISAs for for bovine TGF-β1 and IGF-1. The concentrations of IGF-1 and TGF-β1 in the first milking after calving were 248-1850 ng/ml and 12.4-42.6 ng/ml, respectively, and they declined in correlation with total protein concentration to 27.0-101 ng/ml (IGF-1) and 0.80-3.49 ng/ml (TGF-β1) by the fifth milkings. The amount of TGF-β1 was on average 5.3±1.4% of that of TGF-β2 and there is a high correlation (r=0.966) between the concentrations of these growth factors in the same samples. No free TGF-β1 form of could be detected.

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

INTRODUCTION

Bovine colostrum is a rich source of polypeptide growth factors including insulin-like growth factors (IGF-1 and IGF-2), transforming growth factor-βs (TGF-β1 and TGF-β2) and related molecules (2-5). These growth factors may play an important role, especially in the growth and development of the GI-tract of newborn animals (6-8).

TGF-β1 and TGF-β2 belong to the TGF-β superfamily of growth factors that comprises several related proteins like TGF-β3 (not detected in bovine milk or colostrum), bone

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morphogenic factors, activins, inhibins and Mullerian inhibitor (9-11). TGF-bs have numerous biological functions, although their role in milk is not fully understood. However, evidence suggests that TGF-βs may be involved in the development of gut (12) and regulation of mammary gland (13). In biological fluids TGF-βs exist in inactive (latent)(14) forms, which can be activated by changes in ionic strength, acidification or proteolytic enzymes (15). The major TGF-β form in bovine milk and colostrum is TGF-β2, whereas the rest is TGF-β1 (3, 16).

The major IGF form in bovine colostrum is IGF-1 (2). IGF-1 has been shown to stimulate cell proliferation in GI-tract of newborn piglets (17), and calves (18), but may also have effects on mammary tissue (19). IGF-1 is usually bound to its binding proteins (IGFBP) in biological fluids and IGFBPs have also been detected in bovine milk (20). In IGF-1 assays the growth factor is usually released with an acid-ethanol treatment before analysis (21).

The growth factor assays include both bioassays and immunoassays. In most cases, the bioassays are not suitable for quantitative measurements of growth factors in complex biological fluids like milk and colostrum (15). However, there are highly specific commercial immunoassays (RIA, ELISA) available, but they are usually designed for measuring human growth factors in serum and cell culture media samples.

In this paper we validated commercial ELISAs for bovine TGF-β1 and IGF-1. In addition, we measured the concentration of these growth factors in colostrum samples of the first five milkings after calving.

MATERIALS AND METHODS

Bovine colostrum samples

Colostrum samples from the five first milkings after calving were collected from Ayshire cows. The samples were frozen immediately and stored at -20°C. Before analysis the

samples were centrifuged at 10,000 g for 10 min (Sorvall RC-5B, DuPont, Wilmington, Delaware, USA) to remove lipids.

A pool of the five first milkings was made for validation experiments. Aliquots of the pool were stored at -20°C and each aliquot was thawed only once before use.

TGF-β1 and IGF-1 ELISAs and standards

TGF-β1 and IGF-1 ELISA kits were from R&D Systems Inc. (Abingdon OX 14 3YS, UK) and Diagnostic Laboratories Inc. (Webster, Texas, USA), respectively. The kits have been designed for measuring human TGF-β1 and IGF-1 from serum and cell culture samples.

Natural bovine TGF-β1 standard (NIBSC/WHO interim reference standard 89/516) was from NIBSC (National Institute for Biological Standards and Control, Potters Bar 3QH, UK). The TGF-β1 standard contained 1 500 U/ml (80 ng) according to NIBSC. The TGF-β1 standards of the TGF-β1 ELISA kit contained recombinant human TGF-β1. The IGF-1 ELISA kit contained recombinant human IGF-1 standards, which were calibrated using a WHO reference standard for human IGF-1 from NIBSC (NIBSC 87/518).

RESULTS

TGF-β1 ELISA

Activation of TGF-β1

The inactivated latent TGF-β1 form cannot be detected by the ELISA kit, according to the manufacturer. To measure the total amount of TGF-β1 a sample of the colostrum pool was activated by HCl (final concentration of 1 M) at room temperature for 1 hour according to the instructions of the manufacturer. The acid was neutralized by diluting (at least 1:30) the sample with the sample dilution buffer of the kit before analysis.

TABLE 1.

Linearity of the TGFβ-1 ELISA at dilutions of 1:15-1:240 of an activated sample of the colostrum pool.

Dilution factor	observed (pg/ml)	expected (pg/ml)	Recovery (%)
1:15	292	524	56
1:30	227	262	87
1:60	131	131	100
1:120	60.7	65.5	93
1:240	29.9	32.8	91

Linearity

To determine the linearity of the ELISA and the minimum dilution of the activated samples a dilution series (1:15-1:240) of activated colostrum pool was assayed. The results (Table 1) indicate that the minimum dilution of the activated colostrum samples was 1:30. At a dilution of 1:15, HCl and/or some unknown factors in the sample matrices interfered. In the following experiments the colostrum samples were diluted at least 1:30. No detectable amounts of TGF-β1 was observed in inactivated samples.

Recovery

Recovery studies were performed using both pre- and post-activation protocols. For the pre-activation recovery study, different amounts of purified TGF-β1 (standard of the kit) was added to the samples of the colostrum pool, activated with HCl, diluted in the sample dilution buffer of the kit (neutralization) and assayed. For the post-activation recovery study, colostrum samples were first activated, neutralized and then spiked with purified TGF-β1. The recoveries in the pre- and post-activation study are shown in Tables 2 and 3. The observed recoveries (69-121%) were in the range of those obtained for serum samples (74-125%) by the kit manufacturer.

Intra- and inter-assay precision

7 replicate samples of the colostrum pool were analyzed to determine the intra-assay

TABLE 2.

The pre-activation study. Samples of the colostrum pool were spiked with different amounts of purified TGFβ-1, then activated, neutralized and analyzed.

endogenous (ng/ml)	added (ng/ml)	observed (ng/ml)	expected (ng/ml)	recovery (%)
3.79	2.35	7.08	6.14	115
3.79	4.69	10.1	8.48	119
3.79	9.38	15.3	13.2	116
0.47	2.35	3.41	2.82	121
0.98	2.35	3.81	3.33	114
1.90	2.35	4.46	4.25	105

TABLE 3.

The post-activation study. Samples of the colostrum pool were activated, neutralized and then spiked with different amounts of purified TGF-β1 before analysis.

endogenous (pg/ml)	added (pg/ml)	observed (pg/ml)	expected (pg/ml)	recovery (%)
77.6	62.5	117	140	84
77.6	250	275	328	84
77.6	750	601	828	73
250	155	309	405	76
250	51.7	221	302	73
250	38.8	200	289	69

precision. The inter-assay precisions was based on 6 separate measurements. Intra- and inter-assay precision were 8.8±0.6 ng/ml (CV 6.8%) and 8.0±1.0 ng/ml (CV 13%), respectively.

Standardization

The TGF-β1 ELISA kit contains recombinant human TGF-β1 standards. Only 29 N-terminal amino acids of bovine TGF-β1 has been sequenced and shown to be indentical to the corresponding amino acid sequence of human TGF-β1 (3). Thus, there is a possibility that the monoclonals of the kit do not show 100% cross-reactivity with bovine TGF-β1. Therefore, the assay was standardized using an international natural bovine

TGF-β1 reference preparation (NIBSC 89/516). The preparation contains a standardized amount of TGF-β1 activity. When the preparation was analyzed using the ELISA, it was found that 1.0 ng of the bovine TGF-β1 corresponded to 43.3 U of standardized activity of bovine TGF-β1. As far as we know this is the only bovine TGF-β1 available.

IGF-1 ELISA

Linearity

To determine the linearity of the ELISA and the minimum dilution of samples, dilution series (1:2-1:64) of a sample of the colostrum pool was assayed. The results (Table 4) indicated the assay was linear in the range of these dilutions. Thus, in this assay no matrix effect was observed. The assay contains a standard acid-ethanol extraction step before analysis, which removed a significant portion of material from the samples and released IGF-1 from its binding proteins.

Recovery

The recovery studies were performed using both pre- and post-extraction protocols. For the pre-extraction recovery studies, purified IGF-1 (NIBSC standard 87/518) was added into samples of the colostrum pool, extracted, neutralized and assayed according to the instructions of the manufacturer. For the post-extraction recovery study, colostrum samples were first extracted, neutralized and then spiked with purified IGF-1. The observed recoveries (Tables 5 and 6) were in the range of those (83-111%) obtained for serum samples according to the manufacturer.

Intra- and inter-assay precision

8 replicate samples of the colostrum pool were analyzed to determine the intra-assay precision. The inter-assay precision was based on 7 separate measurements. Intra-assay and inter-assay precisions were 430±31 ng/ml (CV 7.2%) and 375±47 ng/ml (CV 13%), respectively.

TABLE 4.

Linearity of IGF-1 ELISA at dilutions of 1:2-1:64 of a sample of the colostrum pool.

Dilution factor	observed (ng/ml)	expected (ng/ml)	Recovery (%)
1:2	199	199	100
1:4	106	99.5	107
1:8	52.5	49.8	105
1:16	20.5	24.9	82
1:32	8.94	12.4	72
1:64	4.57	6.22	73

TABLE 5.

The pre-extraction study. Samples of the colostrum pool were spiked with different amounts of purified IGF-1, then extracted and analyzed.

Endogenous (ng/ml)	added (ng/ml)	observed (ng/ml)	expected (ng/ml)	recovery (%)
78.1	14.0	95.9	92.1	104
78.1	28.1	94.8	106	89
78.1	56.1	115	134	86
78.1	112	159	190	84

TABLE 6.

The post-extraction study. Samples of the colostrum pool were extracted, then spiked with different amounts of purified IGF-1 and analyzed.

endogenous (ng/ml)	added (ng/ml)	observed (ng/ml)	expected (ng/ml)	recovery (%)
0.810	0.152	1.12	0.962	116
0.810	0.304	1.10	1.11	99
0.810	0.619	1.58	1.42	111
0.810	1.22	2.29	2.03	113

Standardization

According to the manufacturer the IGF-1 ELISA has been standardized using an international reference standard for human IGF-1 from NIBSC (NIBSC 87/518). Since the amino acid sequences of human and bovine IGF-1 are identical (2), the cross-reactivity of

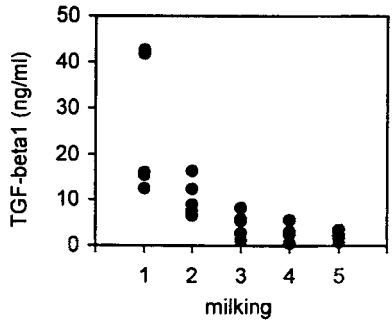

Fig.1 TGF-β1 concentration in the five first milkings of five cows.

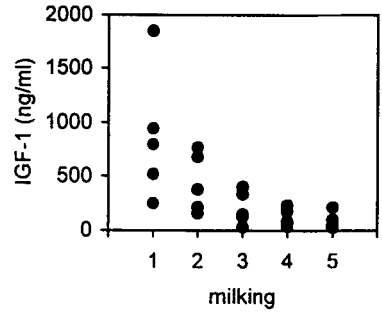

Fig.2 IGF-1 concentration in the five first milkings of five cows.

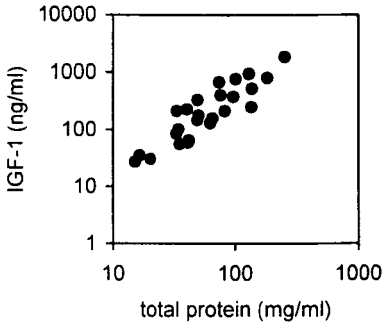

Fig.3 Correlation between IGF-1 and total protein concentrations. Total protein concentrations were measured previously (1). (r=0.886).

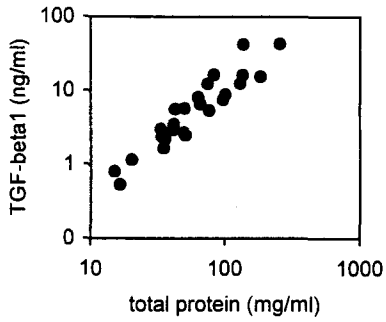

Fig.4 Correlation between TGF-β1 and total protein concentrations. Total protein concentrations were measured previously (1). (r=0.847).

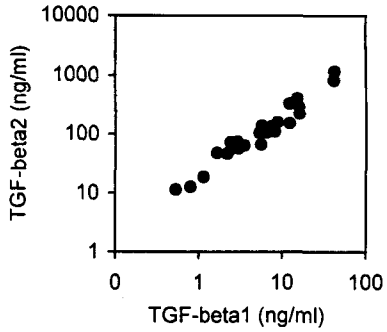

Fig.5 Correlation between TGF-β1 and TGF-β2 concentrations. TGF-β2 concentrations were measured previously (1). (r=0.966).

the antibodies should be 100%. Human IGF-1 has also been generally used as a standard, when bovine IGF-1 has been measured (6).

TGF-β1 and IGF-1 concentrations in bovine colostrum

The concentration of the TGF-β1 and IGF-1 in the first five milkings of colostrum from five Ayshire cows were measured using the above mentioned ELISAs. Previously, the TGF-β2 and the total protein concentration of the same samples were measured (1). The results in Figures 1 and 2 show that the concentration of these growth factors declined

milking by milking and there were significant differences in the concentrations of these growth factors in the first milkings. The correlations between the concentrations of the growth factors and total protein were also analyzed. The results in Figures 3-5 show that there is a very high correlation (r=0.966) between the concentrations of TGF-β1 and TGF-β2 and slightly lower between TGF-β1, IGF-1 and total protein.

DISCUSSION

Milk/colostrum growth factors may play an essential role in the development of the GI-tract of newborn animals. The mostwell known growth factors included TGF-β1, TGF-β2, IGF-1 and IGF-2. Of these growth factors, IGF-1 was carefully studied, since it was obviously the predominant growth factor in bovine milk/colostrum (6-8).

Colostrum/milk IGF-1 and IGF-2 have usually been measured using RIAs (7, 6) and only recently, IGF-1 specific ELISAs have been developed (21), including the assay described in this paper. There are commercial RIAs for human IGF-1, but they are designed for diagnostic purposes to be used in clinical laboratories.

We measured 248-1850 ng/ml IGF-1 in the first milking and the results correspond with the published values of IGF-1 in bovine colostrum (100-2000 ng/ml) (6, 8). IGF-1 concentration declined in correlation (r=0.886) with the total protein concentration to 27.0-101 ng/ml by the fifth milkings. We also analyzed a sample of normal milk and it contained 13.7 ng IGF-1/ml.

We also tested a commercial human IGF-2 ELISA, but the monoclonals in that assay did not show cross-reactivity with bovine IGF-2 (data not shown). The result was also confirmed by the manufacturer. Similar low cross-reactivity between anti-human IGF-2 and bovine IGF-2 has also been reported previously (22), which is interesting, since human and bovine IGF-2 differed by only three amino acids (2). The few published bovine IGF-2 measurements were based on RIAs developed in the corresponding laboratories (23-25).

TGF-β1 is the major form in serum and most tissues including spleen, liver and kidney (26, 27), whereas the predominant TGF-β form in bovine milk (3) and colostrum (16) was TGF-β2 (85-95%). Rogers (28) used a bioassay and measured 4.3 ng TGF-β (total)/ml in bovine milk (28), but as far as we know, no quantitative measurements of bovine TGF-β forms in milk/colostrum have been presented previously excluding our studies.

Although the TGF-β1 ELISA kit used in this study has been designed for determination of human TGF-β1, the validation experiments indicated that the ELISA was suitable for measuring bovine colostrum samples, when the activated samples were diluted at least 1:30. No active TGF-β1 was detected in colostrum samples, whereas it was previously found that about 6% of total TGF-β2 was in an active form in bovine colostrum (1). The TGF-β1 concentration of the first milking after calving was 12.4-42.6 ng/ml and it declines together with total protein concentration (r=0.847) to 0.80-3.49 ng/ml by the fifth milkings. We also analyzed a sample of normal milk and it contained 3.7 ng TGF-β1/ml. Based on the previous TGF-β2 measurements (1), the amount of TGF-β1 was on average 5.3 ± 1.4% of that of TGF-β2 and we found a very high correlation (r=0.966) between the concentration of these growth factors in the colostrum samples.

We have now validated ELISAs for the most well-known bovine milk/colostrum growth factors excluding IGF-2. These assays provide the possibility, to specifically measure, the concentration of these growth factors, which may also give information about their biological functions in milk/colostrum. The assays are freely available and can also be standardized using international growth factor standards from NIBSC. This makes it possible to calibrate these assays with other quantitative analysis methods of these growth factors.

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