Measurement of betacellulin levels in bovine serum, colostrum and milk

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Abstract

Betacellulin, a member of the epidermal growth factor (EGF) family, was originally isolated and identified from the conditioned medium from a murine pancreatic β-cell carcinoma cell line. Recently, we isolated bovine betacellulin from a growth factor enriched cheese whey extract, but there is no information on the presence of betacellulin in other biological fluids. We have cloned the cDNA for bovine betacellulin, produced recombinant betacellulin and shown that it has a similar potency to the purified native molecule in stimulating the proliferation of Balb/c3T3 fibroblasts. We have produced a polyclonal antiserum to bovine betacellulin which did not cross-react with EGF or transforming growth factor-α (TGF-α). The antibody was used in a homologous RIA that was able to detect betacellulin in pooled bovine colostrum sampled during the first 3 days after calving (2·30 ± 0·11 ng/ml mean ± S.E.M.; $n=6$), in bovine milk soluble fraction (1·93 ± 0·64 ng/ml mean ± S.E.M.; $n=5$) and in bovine cheese whey (2·59 ± 0·16 ng/ml mean ± S.E.M.; $n=3$). The betacellulin concentration in foetal bovine serum (FBS) (3·68 ± 0·59 ng/ml mean ± S.E.M.; $n=6$) greatly exceeded that of betacellulin in serum from male calves 1 and 5 weeks of age (0·53 ± 0·15 ng/ml and 0·70 ± 0·09 ng/ml respectively; mean ± S.E.M.; $n=9$). Betacellulin measured in the serum of these same animals when aged between 27 and 43 weeks was below the detection limits of the RIA. Sera from 10 out of 36 unmated heifers contained betacellulin levels within the detection limits of the assay (0·433 ± 0·06 ng/ml mean ± S.E.M.; $n=10$). The presence of betacellulin in bovine colostrum and milk suggests that it plays a role in the growth and development of the neonate and/or mammary gland function. The results also show that betacellulin is undetectable in the castrated adult male circulation. Additionally, although present in very low amounts, serum betacellulin could be under hormonal regulation in the female, since betacellulin was detected in sera from 27% of the unmated heifers examined in this study. The high levels of betacellulin detected in FBS relative to newborn and adult serum suggests a possible endocrine role for this growth factor in the bovine foetus.

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Introduction

Betacellulin was first isolated as a mitogen in the conditioned medium of a mouse pancreatic β-cell carcinoma cell line (Shing *et al.* 1993). Mouse betacellulin is a 32 kDa glycosylated polypeptide composed of 80 amino acids which shares 82·5% and 79% amino acid sequence identity with the human and bovine form respectively (Shing *et al.* 1993, Watanabe *et al.* 1994, Dunbar *et al.* 1999). Betacellulin is one of the epidermal growth factor (EGF) family of growth factors. The other members include amphiregulin, epiregulin, heparin-binding EGF-like growth factor (HB-EGF), neural- and thymus-derived activator for erbB kinases (NTAK), the neuregulin gene products and transforming growth factor-α (Marchionni *et al.* 1993, Carraway *et al.* 1997, Chang *et al.* 1997, Higashiyama *et al.* 1997, Raab & Klagsbrun 1997, Zhang *et al.* 1997). Betacellulin shares significant sequence homology with the other members, including a characteristic six-cysteine consensus motif within the EGF-like domain, which forms three intra-molecular disulphide bonds. Another common feature of the EGF family members is that they are produced as transmembrane precursor molecules and are proteolytically cleaved to give rise to the soluble mature growth factor. Betacellulin is able to bind and activate members of the EGF tyrosine kinase receptor family encoded by the erbB genes, specifically, erbB-1 (EGFR) and erbB-4 (Watanabe *et al.* 1994, Riese *et al.* 1996). It has

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since been demonstrated that betacellulin is not only able to activate homodimers of erbB-1 and erbB-4, but also combinations of erbB receptor heterodimers, including the oncogenic erbB-2–erbB-3 complex (Alimandi *et al.* 1997).

Betacellulin is expressed predominantly in pancreas and small intestine but also weakly in heart, lung, liver, skeletal muscle, kidney, prostate, testis, ovary and colon (Sasada *et al.* 1993, Seno *et al.* 1996). It is a known mitogen for numerous cell types including smooth muscle cells, Balb/c3T3 cells, Madin Darby canine kidney epithelial cells and retinal pigment epithelial cells (Shing *et al.* 1993). Despite this knowledge the biological role of betacellulin *in vivo* is not yet understood.

Bovine milk is rich in a number of growth factors including platelet-derived growth factor (PDGF) and the insulin-like growth factors, IGF-I and IGF-II (Shing & Klagsbrun 1984, Francis *et al.* 1988). Previous studies in this laboratory have produced a novel whey extract that comprises a concentrated mixture of bovine milk proteins enriched in growth factors (Francis *et al.* 1995). This extract is a source of potent growth-promoting activity for a number of mesoderm-derived cell types (Belford *et al.* 1995). A recent study by Dunbar *et al.* (1999) has since found that betacellulin accounts for about 50% of the growth-promoting activity within this extract when tested in a Balb/3T3 bioassay.

The implication of this finding is that betacellulin is present in bovine milk. Taken together with its role as a mitogenic factor for a number of cell types and its expression, along with its receptors in numerous tissues, suggests a major biological role. Receptors for betacellulin have been demonstrated in liver and placental tissue (O'Keefe *et al.* 1974, Wang *et al.* 1992, Mielke *et al.* 1998), which raises the possibility that betacellulin, may be in the circulation. Using a homologous RIA for bovine betacellulin, we were able to confirm the presence and determine the levels of betacellulin in bovine colostrum and milk. Additionally, we demonstrated the presence of betacellulin in serum, and the effects of age and sex of the animal on the serum betacellulin concentration.

Materials and Methods

Production of recombinant bovine betacellulin

A modification of the expression vector p[Met^1]-pGH (1–46)IGF-I (King *et al.* 1992) was used to produce recombinant bovine betacellulin. This plasmid directs isopropyl-D-galactoside (IPTG)-induced high-level expression of betacellulin as a fusion protein that has a 46 amino acid amino-terminal extension derived from methionyl porcine growth hormone (pGH). A modification was made to introduce a methionine in-between the fusion partner and betacellulin so that the fusion protein could be cleaved to release authentic bovine betacellulin, following proteolytic digestion with cyanogen bromide.

The cDNA encoding the complete nucleotide sequence of mature bovine betacellulin [Asp^1-Tyr^{80}] was generated by RT-PCR. Total RNA was isolated from 80–90% confluent Madin Darby bovine kidney cells (MDBK, ATCC CCL 22) and cDNA synthesized from 1 μg total MDBK RNA using oligo dT primer and Superscript II (Life Technologies, Melbourne, Australia). The subsequent cDNA was used as a template for PCR with oligonucleotide primers, 5′ ATC TAG GTT ACC ATG GAT GGG AAT TCA ACC AGA 3′ and 5′ CTA GAT AAG CTT TCA TCA GTA AAA CAA GTC AAC TCT 3′. The resultant 273 bp PCR product was purified, digested with HpaI and HindIII and cloned into the HpaI/HindIII digested expression vector p[Met^1]-pGH(1–46). The recombinant plasmid was maintained in *E. coli*, strain JM101.

The *E. coli* JM101 strain harbouring the plasmid p[Met^1]-pGH(1–46)-Val-Asn-Met-betacellulin was selected as a single colony and used to inoculate a 20 ml starter culture in a medium (K_2HPO_4 60 mM, KH_2PO_4 33 mM, $(NH_4)_2SO_4$ 7·5 mM, sodium citrate 1·7 mM, $MgSO_4.7H_2O$ 10 μM, D-glucose 0·2%, thiamine 0·0005%, ampicillin 50 μg/ml). The culture was grown at 37 °C for 16 h. The starter culture was in turn used to inoculate two 5 l fermenters (Applikon, Schiedam, The Netherlands) each containing 3 l of growth medium (NH_4Cl 30 mM, K_2SO_4 7 mM, KH_2PO_4 12 mM, Na_2HPO_4 19 mM, D-glucose 139 mM, $MgSO_4.7H_2O$ 2·4 mM, thiamine 0·0004%, $Fe_2SO_4.7H_2O$ 35 μM, $MnSO_4.7H_2O$ 7·4 μM, $CuSO_4.7H_2O$ 0·8 μM, trisodium citrate 74 μM and ampicillin 50 μg/ml). Bacteria were grown at 37 °C until the absorbance at 600 nm reached an OD of 4·0 and then induced with IPTG (0·33 mM) and the cultivation was continued until glucose became limiting, indicated by a sharp rise in pH. Regulation of temperature, pH and oxygen was under automatic control (FC4 Data system, Real Time Engineering, Sydney, Australia). Cells were disrupted at 5000 p.s.i. following five passes through a homogenizer (Rannie Instruments, APV Homogenisers, Albertslund, Denmark) and inclusion bodies collected by centrifugation (10 000 r.p.m., 25 min, 4 °C). The inclusion bodies were washed twice with NaCl (30 mM), KH_2PO_4 (10 mM), $ZnCl_2$ (0·5 mM), harvested by centrifugation at 6000 r.p.m. and stored at − 80 °C.

Washed inclusion bodies, in 20 g batches, were thawed, suspended at 10% (w/v) in a buffer containing urea (8 M), Tris–HCl (0·1 M), glycine (40 mM), dithiothreitol (40 mM) and $ZnCl_2$ (0·5 mM) (pH 9·0) and stirred for 30 min at room temperature. The solubilized inclusion bodies were centrifuged at 14 000 r.p.m. for 20 min and the resultant supernatant desalted on a XK column (5 cm × 100 cm; Amersham Pharmacia Biotech, Sydney, Australia) packed with Cellufine GCL-1000 (Chisso Corporation, Tokyo, Japan) and equilibrated with urea (8 M), Tris–HCl (0·1 M), glycine (40 mM), dithiothreitol

(1·6 mM) and $ZnCl_2$ (0·5 mM) (pH 9·0) at a flow rate of 2 ml/min. Thirty millilitre fractions were collected and those containing recombinant [Met[1]]-pGH(1–46)-Met-betacellulin fusion protein were pooled and subject to oxidative refolding by diluting the pool to a final protein concentration of 0·1 mg/ml in buffer containing urea (4 M), glycine (40 mM), Tris–HCl (0·1 M), EDTA (5 mM), dithiothreitol (0·4 mM) and 2-hydroxethyldisulphide (1 mM) (pH 9·0). After stirring for 3 h at room temperature, the reaction was stopped by adjusting the pH to 6·45 with HCl (1 M). The refolded fusion protein was further purified on a S-Sepharose Fast Flow column (5 cm × 15 cm; Amersham Pharmacia Biotech) equilibrated with urea (8 M), ammonium acetate (50 mM) (pH 6·45) at a flow rate of 15 ml/min. The column was washed with the same buffer until the optical density ($OD_{280\ nm}$) returned to baseline. The column was then eluted with a linear gradient of NaCl (0–0·7 M) in the same buffer at a flow rate of 15 ml/min. Fractions of 30 ml were collected and those containing fusion protein pooled. The fusion protein pool was desalted and further purified by HPLC on a C_4 Prep-Pak column (40 mm × 100 mm; 300 Å pore size, 15 μm bead size; Millipore-Waters, Lane Cove, Australia). The protein pool was adjusted to 0·1% trifluoroacetic acid (TFA) and loaded onto the C_4 column at 50 ml/min. The column was washed extensively with 0·1% TFA and protein eluted with a gradient of 18–50% (v/v) acetonitrile over 90 min in the presence of 0·08% TFA at a flow rate of 20 ml/min. Fractions of 30 ml were collected and those containing fusion protein pooled and lyophilized.

To produce authentic bovine betacellulin, the fusion protein was cleaved by solubilizing the lyophilized protein in HCl (0·13 M) containing a 100-fold molar excess of cyanogen bromide at a protein concentration of 10 mg/ml. The cleavage reaction was performed at room temperature in the dark for 25 h. Authentic betacellulin was separated from its fusion partner by HPLC. The cleavage reaction was diluted 1:4 (v/v) with 0·1% TFA and applied to a C_4 Prep-Pak column (Millipore-Waters) at a flow rate of 50 ml/min. The column was washed with 0·1% TFA until $OD_{280\ nm}$ returned to baseline and the column was then eluted with a gradient of 16–32% (v/v) acetonitrile over 150 min in the presence of 0·08% TFA at a flow rate of 20 ml/min. Fractions of 26 ml were collected and those containing pure betacellulin pooled.

Cell culture

Balb/c3T3 fibroblasts (CCL 163) were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM containing 10% foetal bovine serum (FBS), together with 100 mg streptomycin, 60 mg penicillin and 1 mg fungizone per litre of growth medium (CSL Limited, Parkville, Australia). All cells were grown and used for experiments as monolayers at 37 °C in 5% CO_2.

The ability of recombinant bovine betacellulin to promote proliferation of cell monolayers was determined using a 96-well plate dye-binding assay (Oliver *et al.* 1989). Cells were seeded at a density of 2×10^5 cells/ml and incubated overnight in the appropriate media to facilitate cell attachment. Cells were washed thoroughly to remove residual media prior to addition of the indicated concentrations of purified native bovine betacellulin (Dunbar *et al.* 1999) or recombinant bovine betacellulin, which had been diluted in serum-free media containing BSA (Sigma-Aldrich, St Louis, MO, USA, 0·1% (w/v)). A FBS-positive control was included on each plate. Cells were incubated in the presence of growth factor for 48 h, washed twice with NaCl (150 mM), fixed with methanol, stained with 1% (w/v) methylene blue (Sigma-Aldrich) and the absorbance read at 655 nm. Data are expressed as the percentage of the growth response observed in serum-free medium and plotted using SigmaPlot version 4·0 (Jandel Scientific, San Rafael, CA, USA).

Antibody production

Antibody production was approved by the Animal Ethics Committee of the Women's and Children's Hospital, Adelaide, SA, Australia. Three female New Zealand semi-lop rabbits were obtained from the Institute of Medical and Veterinary Sciences (Gilles Plains, SA, Australia). Each rabbit was injected subcutaneously at five different sites around the shoulder and neck with 500 μg of recombinant bovine betacellulin in Freund's complete adjuvant (Sigma-Aldrich). After 6 weeks, animals were given a first booster injection of 100 μg betacellulin in Freund's incomplete adjuvant (Sigma-Aldrich) and a similar, second boost, 4 weeks later. Blood samples were obtained 2 weeks after each booster injection for determination of antibody titre. Animals were sacrificed 4 weeks after the second booster and blood was collected by cardiac puncture.

RIA

Recombinant bovine betacellulin was iodinated with carrier-free Na[^{125}I] (Amersham Pharmacia Biotech), to a specific activity of 65 μCi/μg using a modification of the chloramine T method (Gargosky *et al.* 1990).

A betacellulin standard curve was prepared by dilution of a frozen stock solution (50 ng/ml). The range of the standard curve was from 4·9 to 5000 pg/tube. Each tube contained either 100 μl of assay buffer (NaH_2PO_4 30 mM, protamine sulphate 0·2% (w/v), disodium EDTA 10 mM, NaN_3 0·2% (w/v), Tween-20 0·05% (w/v), pH 7·5), 100 μl sample or 100 μl standard in assay buffer, 25 μl ^{125}I-labelled betacellulin (approximately 20 000 c.p.m.) in assay buffer and 25 μl of betacellulin antiserum (1:50 000)

in assay buffer (final dilution 1:300 000). The tubes were mixed and incubated for 16 h at 4 °C. A 50 µl aliquot of goat anti-rabbit IgG (GroPep Limited, Adelaide, Australia) in assay buffer and 10 µl of rabbit immunoglobulin (Dako Corporation, Carpinteria, CA, USA) pre-diluted 1:200 v/v in assay buffer, were added to each tube and incubated for a further 30 min at 4 °C. Precipitation of bound betacellulin was assisted by the addition of 1 ml ice-cold polyethylene glycol 6000 (6% (w/v) in 150 mM NaCl) and the tubes centrifuged at 4000 ***g*** for 20 min at 4 °C. The supernatant was removed by aspiration and radioactivity in the remaining pellet measured using a Model 1470 Gamma Counter (Wallac Oy, Turku, Finland). The concentration of betacellulin in each tube was calculated using RIAcalc software (Wallac Oy). Unknown samples and standards were measured in triplicate.

Sample preparation

The nine Friesian Holstein bulls used in this study were castrated at 10 weeks of age and their blood samples collected by jugular venipuncture on several occasions between 1 and 43 weeks. These animals were maintained at the University of Queensland, Pastoral and Veterinary Centre, Goondiwindi, NSW, Australia and were fed Ultragrow calf milk replacer (Millmaster Feeds, Tamworth, NSW, Australia), had access to hay and allowed to feed *ad libitum*, and were offered Ultragrow calf pellets (Millmaster Feeds) once daily for the first 6 weeks before being weaned off milk replacer. The Animal Ethics Committee of the University of Queensland approved the sample collection. Blood samples were also collected by coccygeal venipuncture from 36 unmated Friesian Holstein heifers with a mean age of 50·5 ± 2·0 weeks (mean ± S.E.M.) from the Roseworthy Campus dairy herd, University of Adelaide. The Animal Ethics Committee of the University of Adelaide approved the sample collection. Blood samples were allowed to clot at 4 °C for 16 h, centrifuged at 4000 ***g*** at 4 °C for 20 min and the serum collected for analysis. Six distinct batches of FBS for measurement of betacellulin concentration were obtained from Trace Scientific (Melbourne, Australia).

Bovine colostrum and milk were collected from cows with an average age of 225·26 ± 49·54 weeks (mean ± S.E.M.; $n=11$). Colostrum from six cows was collected at the morning and afternoon milking for the first 3 days after calving and stored at − 20 °C. Samples were prepared for measurement of betacellulin using an adaptation of the method of Francis *et al.* (1988). Samples were thawed, centrifuged at 21 000 ***g*** for 30 min at 4 °C and the resulting infranatant mixed with 0·33 volumes of Freon[1,1,2-trichloro-1,2,2,-trifluoroethane] (Dupont Wilmington, MA, USA) for 15 min. A further centrifugation step performed at 4000 ***g*** for 15 min at 4 °C, yielded a clear supernatant that was subsequently adjusted to pH 4 with HCl (1 M). The acidified supernatant was centrifuged again for 30 min at 21 000 ***g*** at 4 °C and the resultant supernatant neutralized with NaOH (1 M). Milk from five different cows was taken more than 1 week after calving and used to prepare bovine milk soluble fraction (BMSF). Separated, clarified and pasteurized bovine cheddar cheese whey was sourced from National Foods (Murray Bridge, SA, Australia). Preparation of BMSF and bovine cheddar cheese whey samples for RIA was achieved using the same method used for preparation of colostrum. Total protein content in colostrum, BMSF and whey was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Results

Analysis of the final betacellulin protein pool by microbore C_4 reverse-phase HPLC identified a single protein peak (Fig. 1). The purity of the betacellulin preparation was further confirmed by N-terminal sequence analysis which concurred with the predicted sequence for bovine betacellulin (Dunbar *et al.* 1999) with an approximate purity of >99%. The molecular mass of recombinant betacellulin determined by electrospray ionization mass spectrometry was 8995·1 ± 0·83. This is consistent with the theoretical mass of 8995·02 calculated from the betacellulin amino acid sequence. Following SDS-PAGE analysis, a single protein band of approximately 9 kDa was detected under reducing or non-reducing conditions (Fig. 1, inset).

The biological activity of purified native bovine betacellulin (Dunbar *et al.* 1999) was compared with that of recombinant bovine betacellulin in Balb/c3T3 fibroblasts. Recombinant betacellulin stimulated the proliferation of Balb/c3T3 cells in a dose-dependent manner similar to the native betacellulin (Fig. 2). Half maximal stimulation of Balb/c3T3 cell proliferation was observed at 1·98 ng/ml and 3·29 ng/ml for native betacellulin and recombinant betacellulin respectively.

Antibody dilution curves were constructed by diluting each individual antiserum in assay buffer and measuring its ability to bind ^{125}I-betacellulin. All rabbits raised a highly significant antibody response against recombinant bovine betacellulin. Only one antiserum was chosen for use in the development of a RIA, based on the half-maximum binding of the label at a dilution of 1:300 000 and the absence of any cross-reactivity to related ligands. The antiserum was able to detect recombinant bovine betacellulin to a much greater degree than recombinant human betacellulin (R&D Systems, Minneapolis, MN, USA) by Western immunoblotting (results not shown). The competition between bovine betacellulin, recombinant human EGF and recombinant human transforming growth factor-α (TGF-α) (GroPep Limited) with ^{125}I-betacellulin for binding sites on the polyclonal antiserum raised against recombinant bovine betacellulin is shown in Fig. 3*a*. Half

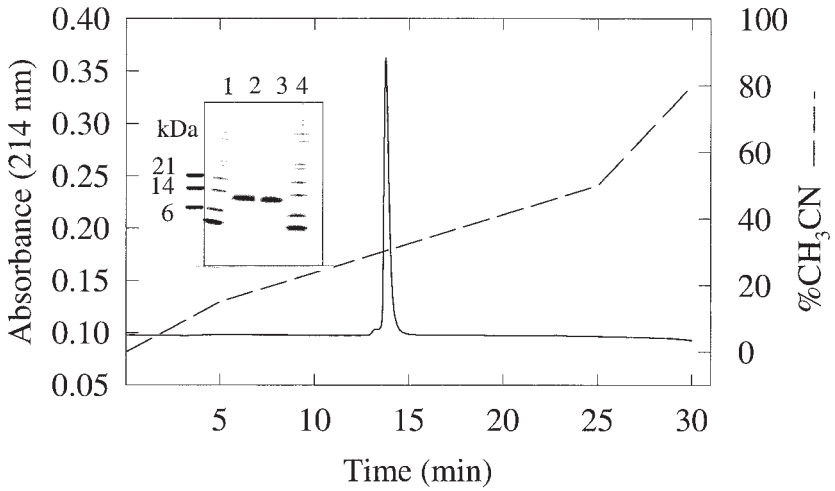

Figure 1 Reverse-phase HPLC of purified recombinant bovine betacellulin. Inset: SDS-PAGE analysis of the purified preparation under reducing (lane 2) or non-reducing conditions (lane 3); lanes 1 and 4, molecular weight markers.

maximal displacement for bovine betacellulin occurred at 0·78 ng/ml. There was no cross-reactivity with human EGF and human TGF-α. The displacement curve for bovine betacellulin was compared with that of serial dilutions of FBS in the RIA. This demonstrated that the serum dilution curve was parallel to the betacellulin standard curve (Fig. 3*b*). Similar dilution curves of colostrum and milk samples were also parallel to the standard curve (results not shown). The coefficients of variation within and between assays for bovine betacellulin were 3·95% and 8·50% respectively.

Colostrum sampled from each of the five animals on days 1, 2 and 3 after calving was pooled according to day of sampling, processed as described and the betacellulin levels measured by RIA. The concentration of betacellulin in day 1, 2 and 3 colostrum pools did not differ significantly (Table 1) although, as expected, the total protein content of colostrum decreased significantly between day 1 and the day 2 and 3 samples ($P<0·001$). When expressed as a percentage of total colostral protein, betacellulin levels increased significantly over the 3 days. Bovine milk soluble fraction and bovine cheese whey had levels of betacellulin similar to that found in colostrum (Table 1).

The betacellulin concentrations in six different batches of FBS were determined using the RIA (Table 1). The mean ± S.E.M. betacellulin concentration for FBS was 3·68 ± 0·59 ng/ml. This was significantly higher than in serum pooled from calves at 1 week and 5 weeks of age, which were 0·53 ± 0·15 ng/ml and 0·70 ± 0·09 ng/ml respectively (Fig. 4). The serum betacellulin concentration in these same animals when aged between 27 and 43 weeks was below the minimum detection limit of the assay (indicated by a solid horizontal line across the graph). Sera from 10 out of the 36 unmated heifers contained betacellulin levels within the detection limits of the assay (0·433 ± 0·06 mean ± S.E.M., $n=10$) (Fig. 4). The results demonstrate that using this homologous RIA, betacellulin is present at high levels in FBS but is undetectable in serum from male cattle over 5 weeks of age. Interestingly, only 27% of the unmated female animals tested contained detectable levels of serum betacellulin.

Discussion

We believe that this is the first study to report the presence of betacellulin by homologous RIA in bovine colostrum and milk. Other members of the epidermal growth factor family have been detected in the colostrum and milk of several species. Specifically, studies have reported the presence of EGF in human (Jansson *et al.* 1985, Read *et al.* 1985, Iacopetta *et al.* 1992), pig (Jaeger *et al.* 1987), rat (Raaberg *et al.* 1990), mouse (Grueters *et al.* 1985) and wallaby (Ballard *et al.* 1995) colostrum and milk and TGF-α in human milk (Connolly & Rose 1988, Okada *et al.* 1991).

Of more relevance to the present study is the earlier finding where EGF activity was detected in bovine colostrum and milk. However, unlike human milk where EGF is a predominant growth factor (Carpenter 1980), PDGF, IGF-I and -II rich bovine milk (Shing & Klagsbrun 1984, Francis *et al.* 1988) appears to contain very little EGF (Iacopetta *et al.* 1992). On average, the colostrum samples analysed in the present study contained approximately 2·3 ng/ml betacellulin, whereas EGF levels of approximately 6 ng/ml have been previously reported (Read *et al.* 1984, Iacopetta *et al.* 1992). EGF concentrations reported in milk vary considerably, most probably

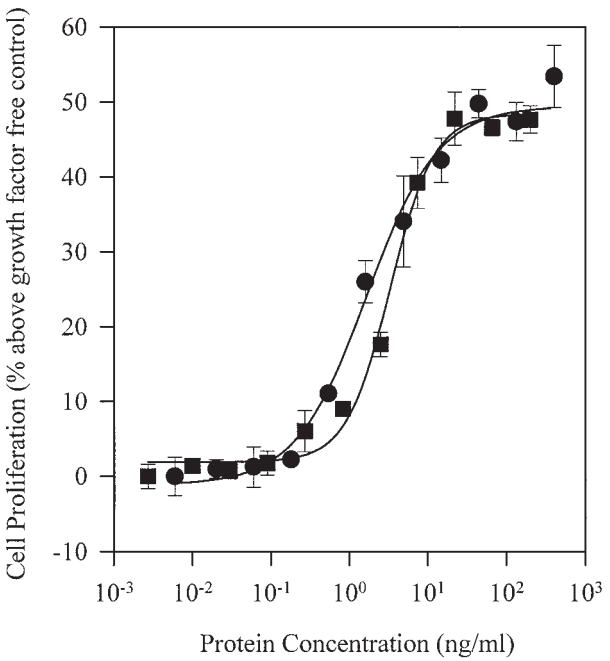

Figure 2 Proliferation of Balb/c3T3 cells in response to purified native bovine betacellulin (●) or recombinant bovine betacellulin (■). Cells were seeded for 16 h in media supplemented with FBS. They were washed in serum-free media, followed by addition of the indicated concentrations of growth factor in serum-free media. After a further 48 h incubation, cell density was measured using a methylene blue dye-binding assay. Results are expressed as the percentage increase in absorbance at 655 nm above the growth factor free control. Data points represent the means of triplicate determinations and error bars indicate the S.E.M. where greater than the symbol size.

as a result of the use of different assay systems. Using a radioreceptor assay with a human lung fibroblast cell line as a source of EGF receptors, Read *et al.* (1984) determined that pasteurized bovine milk contained approximately 2·5 ng/ml EGF. In another study using placental plasma membranes, Yagi *et al.* (1986) estimated that bovine milk contained 320 ng/ml EGF. Subsequent studies by Iacopetta *et al.* (1992) showed that radioreceptor assays performed with placental plasma membranes to measure EGF in bovine milk produced unreliable results, but using the A431 cell line found that bovine milk contained less than 2 ng/ml EGF. Radioreceptor assays can detect proteins with EGF receptor binding capacity, but are limited in that they are unable to discern which specific EGF receptor ligand is being measured. The current homologous RIA uses an antibody that we have shown to be specific for betacellulin and using this assay we found that bovine milk contains approximately 1·93 ng/ml betacellulin. To our knowledge, no previous studies have definitively proven the presence of EGF in bovine milk. Therefore, we suggest that bovine milk does not contain EGF but instead low levels of betacellulin, and that this growth factor may be responsible in part for the bovine milk EGF receptor binding activity detected in the studies by Read *et al.* (1984) and Iacopetta *et al.* (1992).

Concentrations of other growth factors in bovine colostrum such as IGF-I, IGF-II and insulin decline substantially coincident with the fall in milk protein as early lactation proceeds (Malven *et al.* 1987, Vega *et al.* 1991, Lee *et al.* 1995). In contrast, studies in human milk found that TGF-α levels remained fairly stable for 7 days post-partum (Okada *et al.* 1991). Similarly, we have demonstrated that despite the decrease in total protein, colostrum betacellulin levels remain stable for the first 3 days after calving and that similar betacellulin levels occur in mature milk. Francis *et al.* (1995) have shown that bovine milk-derived growth factors are retained in the whey fraction. Our present studies have confirmed this finding and shown that betacellulin concentrations in separated, clarified and pasteurized whey are similar to that in colostrum and milk. The relative stability of the levels of this growth factor throughout bovine lactation suggests a role for betacellulin in the suckling neonate and in mammary function that could be major, and quite distinct from EGF.

A number of studies indicate that milk-derived EGF is required as a regulator of postnatal gut development in the suckling young of rats, mice and pigs (Berseth 1987, Popliker *et al.* 1987, Shen & Xu 1996, 1998), and influences the growth and development of the post-natal liver (McCuskey *et al.* 1997), spontaneous intestinal bacterial translocation in newborns (Okuyama *et al.* 1998) and neonatal eyelid opening (Okamoto & Oka 1985). The significance of betacellulin occurring in bovine milk for the growth and development of the suckling neonate is presently unknown, however, accumulating evidence supports a role for betacellulin in gut development as well. The erbB receptors known to bind betacellulin have been described in various sections of the gut and in gastrointestinal cancers (Quirke *et al.* 1989, Jankowski *et al.* 1992, Prigent *et al.* 1992, Kataoka *et al.* 1998, Noguchi *et al.* 1999), while betacellulin itself is expressed in adult gastrointestinal tract (Seno *et al.* 1996) and increases DNA synthesis in RIE cells (Barnard *et al.* 1994). We have preliminary evidence to show that betacellulin can stimulate proliferation of rat IEC-6 cells *in vitro* and gastrointestinal epithelial cells *in vivo* following systemic administration to rats.

Current knowledge also supports a potential role for betacellulin in mammary development and/or function. Transgenic animal studies have revealed that other members of the EGF family, including TGF-α, amphiregulin and EGF, have essential roles in ductal morphogenesis and lactogenesis in mice (Luetteke *et al.* 1999). erbB receptors have been identified within mammary glands from numerous species including cows (Spitzer & Grosse 1987), and Dunbar *et al.* (1999) have shown that betacellulin mRNA is expressed in bovine mammary tissue.

Betacellulin stimulates the MDA-MB453 mammary epithelial cell line to differentiate and produce milk-like droplets (Pinkas-Kramarski *et al.* 1998). Colostrum or milk-borne betacellulin could be derived from the numerous cells present within these fluids, the maternal circulation or from synthesis within the mammary gland itself. The source of betacellulin in milk, similar to EGF, is unlikely to arise from serum because lactating serum contains much lower levels than milk (S E P Bastian, unpublished results). Since betacellulin is expressed in mammary tissue, local synthesis could contribute to milk betacellulin.

Table 1 Betacellulin and total protein concentrations of FBS, pooled bovine colostrum, bovine milk soluble fraction (BMSF) and bovine cheddar cheese whey

	Betacellulin concentration Mean ± S.E.M. (ng/ml)	**Total protein** Mean ± S.E.M. (mg/ml)
FBS	3·68 ± 0·59	
Colostrum day 1	2·61 ± 0·41	54·5 ± 2·5
Colostrum day 2	2·14 ± 0·17	12·2 ± 0·8*
Colostrum day 3	1·97 ± 0·12	8·6 ± 1·1*
BMSF	1·93 ± 0·64	4·1 ± 0·9
Whey	2·59 ± 0·16	5·1 ± 0·8

*Significantly different to protein concentration in Day 1 colostrum ($P<0.001$).

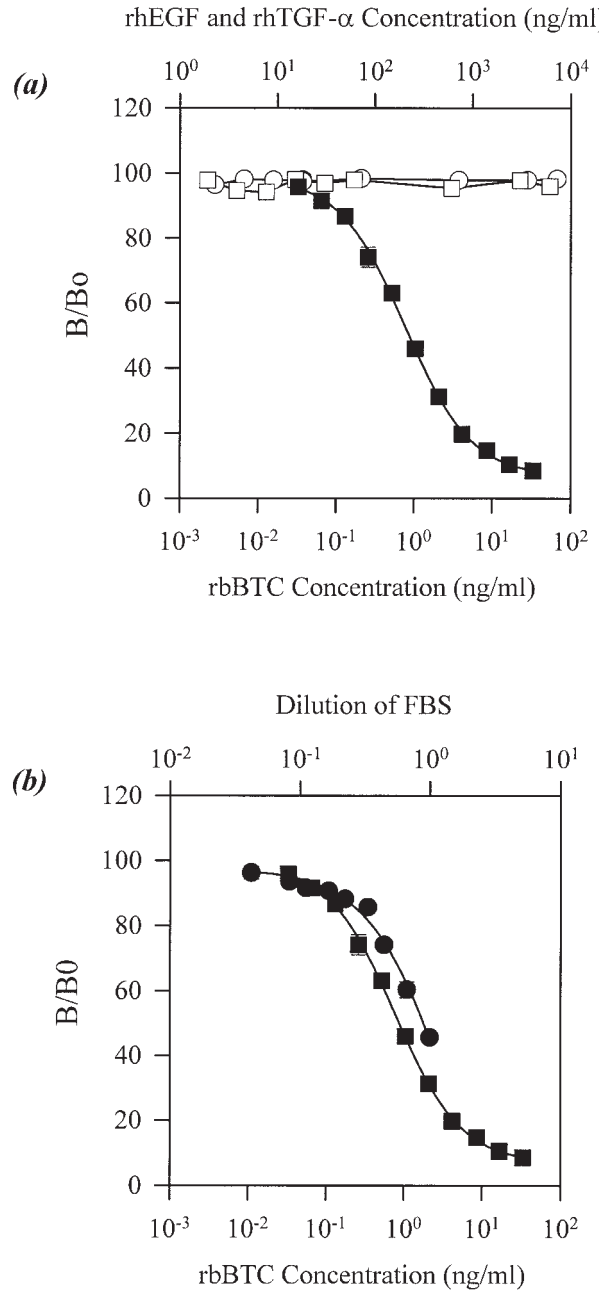

Figure 3 Radioimmunoassay of betacellulin; values are means ± S.E.M.S of triplicate determinations and are expressed as the fraction of ^{125}I-betacellulin bound in the absence of competing ligand (B/B_o). Non-specific binding in the absence of antibody (~1% of total radioactivity) has been subtracted. The competing ligands were (*a*) recombinant human EGF (□) and TGF-α (○) and (*b*) competition observed in the presence of increasing dilutions of FBS (●). Each graph also shows the betacellulin standard curve (■).

The fact that the erbB receptor profile allowing specificity to betacellulin is found in a wide variety of foetal and adult tissues (Quirke *et al.* 1989, Prigent *et al.* 1992) and also in some tumours (Jankowski *et al.* 1992, Fulop *et al.* 1998, Lee and Maihle 1998) would suggest a significant but an as yet undefined role for betacellulin. Although many biological effects of betacellulin may be due to autocrine or paracrine interaction with receptors, the presence of milk-derived betacellulin suggested that it could be in the circulation. We were not aware of any previous studies that had determined blood betacellulin levels, although a number of studies have reported the presence of EGF in plasma and serum of various species (Perheentupa *et al.* 1985, Savage *et al.* 1986). The concentration of betacellulin was approximately eightfold higher in FBS than in newborn serum from 1- and 5-week-old bull calves. This suggests that betacellulin may be an important foetal endocrine growth factor. Male calves contained detectable serum betacellulin at 1 and 5 weeks of age, but betacellulin concentrations were undetectable in the same animals after 27 weeks of age. Newborn calves were fed milk replacer with a 24% minimum crude protein composition. This crude protein was derived from whole milk powder and whey powder concentrate. Gastrointestinal absorption of orally administered ^{125}I-EGF has been clearly demonstrated in suckling rats (Thornburg *et al.* 1984). Since we have shown that

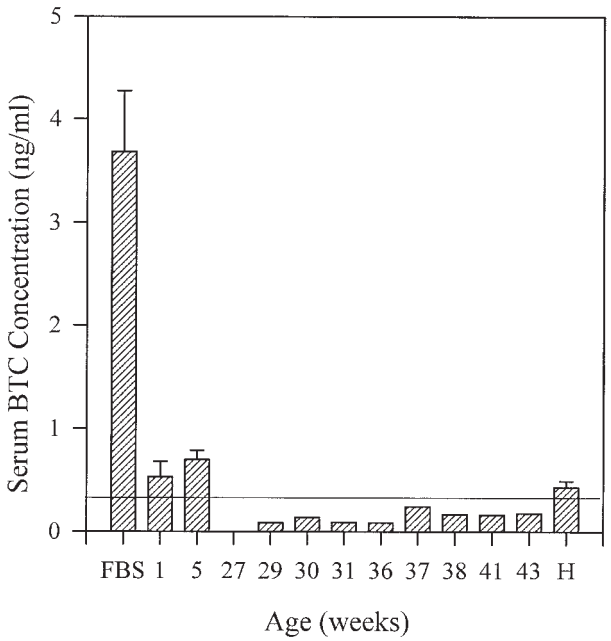

Figure 4 Radioimmunoassay of betacellulin levels in six different batches of FBS, serum from nine steers aged from 1 to 43 weeks and serum from 36 unmated heifers. Data represent the means ± S.E.M.S of triplicate determinations. The numbers 1–43 represent the age in weeks of the nine steers at the time of blood collection. The values are shown only for the ten unmated heifers whose serum betacellulin levels were above the minimum detection limit of the assay (H).

milk and whey contain considerable levels of betacellulin, it is possible that the ingested milk replacer could have acted as a source of serum betacellulin in these newborn calves. Alternatively, it is possible that newborn calves produce endogenous betacellulin, but that synthesis decreases due to increasing age, castration or increased clearance from plasma. The data also indicate that gender affects serum betacellulin concentration. The observation that only 27% of the female animals contained detectable serum betacellulin levels, may indicate that betacellulin could be under control of hormones that regulate oestrus. Studies of EGF blood levels in other species have also reported effects of ontogeny and gender (Perheentupa *et al.* 1985).

Our findings show that significant levels of betacellulin are present in FBS and that betacellulin is also contained in bovine colostrum, mature milk and cheddar cheese whey. They also indicate that in bovine milk, what was once thought to be EGF might in fact be betacellulin. Although we have not categorically ruled out the possibility that a bovine homologue of another member of the epidermal growth factor family may be responsible for the current results, we think this is unlikely. Since the antibody has a low cross-reactivity to human betacellulin, which shares 88% sequence homology with bovine betacellulin, it would be unlikely to recognize other bovine EGF family ligands with a lower homology. Although bovine TGF-α has been cloned and sequenced (Zurfluh *et al.* 1990) we do not believe the peptide is available to test against this betacellulin antibody. The cross-reactivity data indicated that the antibody did not recognize human EGF or human TGF-α. Other commercially available EGF ligands are derived from human sequences and, for the reason outlined above, were not tested.

Expression of receptors for betacellulin has been documented in a diverse variety of cell types in several animal species in foetal and neonatal life. These findings lend credence to the hypothesis that betacellulin may be a modulator of foetal and neonatal growth and development. The true significance of betacellulin in bovine milk and foetal serum and the regulation of betacellulin in adult serum require further study.

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