Journal of Animal and Veterinary Advances 3 (5): 278-284, 2004

Detection and Characterization of Soluble Betaglycan in Porcine Milk

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Abstract: It is well known that animal milk contains various bioactive compounds, such as immunoglobulins, hormones and growth factors. The present study reports the detection of a new bioactive compound, betaglycan, in porcine milk. The concentration of betaglycan in the milk, determined by a specific enzyme-linked immuno-sorben assay, ranged between 0.25 and 0.41 mg ml^{-1}. The concentration was highest in the colostrum and it declined with the progress of lactation. Further studies with Western blot analysis or gel filtration in combination with deglycosylation enzyme treatment demonstrated that the compound consisted of glycosaminoglycan chains and a core protein of about 110 kDa. Pre-treatment of the milk samples with the reducing agent, 2-mercaptoethanol, revealed that the core protein contained disulfide bonds. These findings indicate that the soluble betaglycan detected in porcine milk has a property similar to that of the ectodomain of the membrane-anchored betaglycan. It was also observed that the soluble betaglycan detected in porcine milk was capable of binding TGF-β. It is speculated that the milk-borne betaglycan may play a role in modulating TGF-β activity; the latter has also been reported in porcine milk.

Key words: betaglycan, milk, pig, TGF-beta

Introduction

Betaglycan is a membrane-anchored proteoglycan with a high binding affinity to transforming growth factor beta (TGF-β). The compound is also known as type III TGF-β receptor and is involved in TGF-β signal transduction (Massague, 1998). TGF-β mediates its biological effects through three high-affinity cell surface receptors, the TGF-β type I, type II and type III receptors. Evidence from kinetic studies of ligand-receptor association indicates that TGF-β signal transduction involves the formation of a cell-surface complex consisting of one type III receptor (betaglycan), two TGF-β molecules and four type II receptors prior to the recruitment of the type I receptor (De Crescenzo *et al.*, 2001). It has been shown that increase in betaglycan expression enhances cellular responsiveness to TGF-β (Blobe *et al.*, 2001). Mouse embryos with betaglycan deficiency through gene disruption developed lethal proliferative defects in the heart and apoptosis in the liver during midgestationand primary fibroblasts generated from betaglycan-null embryos exhibited reduced sensitivity to TGF-β2 (Stenvers *et al.*, 2003).

In addition to the membrane-bound betaglycan, a soluble form of betaglycan has been detected in various biological fluids, including culture media of certain cell lines, serum and extracellular fluids (Andres *et al.*, 1989) and rat milk (Zhang *et al.*, 2001). The soluble betaglycan has the structural properties similar to the membrane-bound betaglycan, except that soluble betaglycan lacks a membrane anchor and does not associate with liposomes (Andres *et al.*, 1989). The soluble betaglycan may be originated from the hydrolysis of membrane-bound betaglycan or be released by cells as a separate product. It has been suggested that soluble betaglycan is generated at least in part by cleavage at the site of Lys-Lys sequence near the transmembrane region of the membrane-bound betaglycan (Lopez-Casillas *et al.*, 1991). The physiological role of soluble betaglycan is unclear. It is suspected that soluble betaglycan may modulate TGF-β activity through interfering the association of TGF-β with its receptors. It has been shown that recombinant soluble betaglycan inhibited cellular response to TGF-β in vitro (Nomura *et al.*, 2002)and overexpression of betaglycan by gene transfection reduces tissue response to TGF-β in vivo (Liu *et al.*, 2002). The ratio between soluble and membrane-bound betaglycan is probably an important determinant of TGF-β activity. Earlier studies in our laboratory have shown that porcine milk contains various growth factors including TGF-β (Xu *et al.*, 1999 and Xu, 2003). In this study we report the detection and characterization of soluble betaglycan in porcine milk.

Materials and Methods

Collection and Preparation of Milk Samples: Porcine milk samples were collected from four Large White x Landrace sows at day 1, day 3, day 7 and day 14 of lactation. All milk samples were centrifuged at 10000g for 30 minutes at 4°C to remove lipid. The protein concentration in the milk samples was determined by Lowry's method (Lowry *et al.*, 1951). The samples were then divided into aliquots and stored at -70°C for further analysis.

Enzyme-linked Immunosorbent Assay: The concentration of betaglycan in milk samples was determined by enzyme-linked immunosorbent assay (ELISA) as described by Bandyopadhyay *et al.* (2002). Briefly, milk samples were

coated to the bottom of a 96-well microplate at 4°C overnight. The plate was blocked with 1% bovine serum albumin (BSA) at room temperature for 2 hoursand was then incubated in sequence with goat anti-human betaglycan antibody (Santa Cruz Biotechnology, CA, USA) and donkey anti-goat immunoglobulin antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, CA, USA) at room temperature for two hours respectively. The plate was thoroughly washed at each change of incubation reagents with phosphate-buffered saline (0.01M, pH7.4) containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA). Finally, 100 μl horseradish peroxidase substrate (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich, St Louis, MO, USA) was added to each well. The color development was allowed for 20 minutes and was then stopped by addition of 50 μl 2M H_2SO_4. Recombinant human betaglycan (Santa Cruz Biotechnology, CA, USA) was used as the standard. The intensity of the color reaction was determined with microplate reader at 450 nm with reference wavelength set at 595 nm.

Betaglycan and TGF-β Binding Assay: To test if betaglycan in porcine milk is capable of binding TGF-β, a solid phase binding assay was performed. Briefly, a 96-well microplate was first coated with recombinant human TGF-β1 (Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight. The plate was then blocked with 1% bovine serum albumin (BSA) at room temperature for 2 hours. Milk samples were then added to the coated plate and incubated at room temperature for 2 hours. After extensive wash with phosphate buffered saline (0.01M, pH7.4 containing 0.05% Tween-20), the plate was incubated in sequence with the primary anti-betaglycan antibody, the secondary antibody conjugated with horseradish peroxidase and peroxidase substrate (3,3',5,5'-tetramethylbenzidine) as described previously for the enzyme-linked immunosorbent assay. The color development was allowed for 20 minutes and was then stopped by addition of 50 μl 2M H_2SO_4. The intensity of the color reaction was determined with microplate reader at 450 nm with reference wavelength set at 595 nm.

Western Blot Analysis: The structural properties of betaglycan in the milk samples were characterized by Western blot analysis. Briefly, milk samples were mixed with four volume of the sample buffer (50 mM Tris-HCl, pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol and 0.05% bromohenol blue and boiled for 5 minutes. To test if the milk-borne betaglycan contains disulfide bonds, the sample buffer was added with a reducing agent of 5% 2-mercaptoethanol. The prepared samples were then loaded to 7.5% sodium dodecyl sulfate polyacrylamide gel and electrophoresed at 120 V for 2 hours using an electrophoresis unit (Mini-Proteian II Cell, Bio-Rad, CA, USA). Separated proteins were then transferred to nitrocellulose membrane (0.45 ìm pore size, Pharmacia Biotech, CA, USA) at 100V/250mA for 150 min at 4°C. The nitrocellulose membrane was then blocked by incubation for 2 hours in tris-buffered saline (pH 7.5) containing 3% BSA and 0.1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA). The membrane was then incubated for 2 hours at room temperature in the solution of primary antibody against betaglycan (Santa Cruz Biotech, CA, USA) followed by another 2-hour incubation at room temperature in the biotin conjugated secondary antibody solution (Santa Curz Biotech, CA, USA). After washing with tris-buffered saline, the membrane was incubated for 2 hours at room temperature in the solution of streptavidin-biotinylate horseradish peroxidase complex (Amersham Pharmacia Biotech, Uppsala, Sweden) followed by the incubation for 30 minutes at room temperature in the substrate solution of 0.2 mg ml^{-1} 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) containing 0.1% H_2O_2. The molecular weights of the immunoreactive bands were determined using a molecular weight standard (Invitrogen, CA, USA).

Gel Filtration: The molecular characteristics of the milk-borne betaglycan were further analyzed with neutral size-exclusion chromatography. Milk samples were loaded to a chromatographic column packed with G-200 Sephadex gel (Sigma-Aldrich, St Louis, MO) and eluted in phosphate-buffered saline (0.01M, pH7.4) at a flow rate of 8 ml per hour. Fractions of elute were collected every eight minutes and were determined for protein content and betaglycan concentration. The protein content of the elute fractions was determined by Lowry's method (Lowry *et al.*, 1951). The betaglycan concentration was determined by ELISA method as described previously. The column was calibrated with a gel filtration calibration kit containing thyroglobulin (669-kDa), ferritin (440-kDa), catalase (232-kDa) and aldolase (158-kDa) (Amersham Pharmacia Biotech, Uppsala, Sweden).

Enzymatic Deglycosylation: To test if milk-borne betaglycan contains glycoslyaminoglycan chains, milk samples were incubated at 37°C overnight with 20 mU chondroitinase ABC (Calbiochem, USA), 2 mU heparitinase (Calbiochem, USA), or both of the enzymes in a digestion buffer containing 0.1 M Tris-HCl and 0.03 M sodium acetate (pH 8.2). The digestion was terminated by heating the sample at 100°C for 5 minutes. The samples were then further analyzed by Western blot and gel filtration.

Results

Concentration and TGF-β Binding Ability of Betaglycan in Milk: Betaglycan was detected in all milk samples by

ELISA with a specific antibody. The concentration of betaglycan was highest in the milk collected during the first day of lactationand the concentration declined progressively by the 14th day of lactation (Table 1). When expressed as per unit milk protein, the concentration become relatively constant in milk collected between 3rd and 14th day of lactation, but the concentration was markedly lower in milk collected during the first day of lactation.

The results of the solid phase binding assay showed that betaglycan in porcine milk is capable of binding TGF-β (Fig. 1). The negative control wells initially coated with bovine serum albumin showed negligible color change after incubation with porcine colostrum or mature milk. In contrast, wells initially coated with human recombinant TGF-β1 showed significant color changes following incubation with porcine day-1 or day-3 milkand the color changed in a dose-dependent manner. At the same protein concentration, day-3 milk produced much greater color change when compared with that of day-1 milk (Fig. 1).

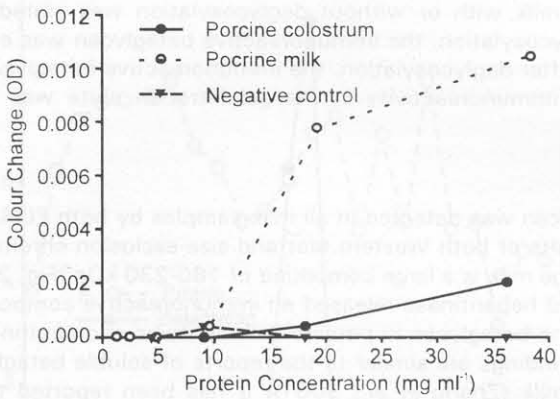

Fig. 1: Binding of milk-borne betaglycan to TGF-β. The wells of a micro-plate were initially coated with recombinant TGF-β1 or bovine serum albumin (negative control) and then incubated with porcine colostrum (day-1 milk) or mature milk (day-3 milk) diluted to various protein concentrations. The amount of betaglycan bound to the well was determined by ELISA as described in the text

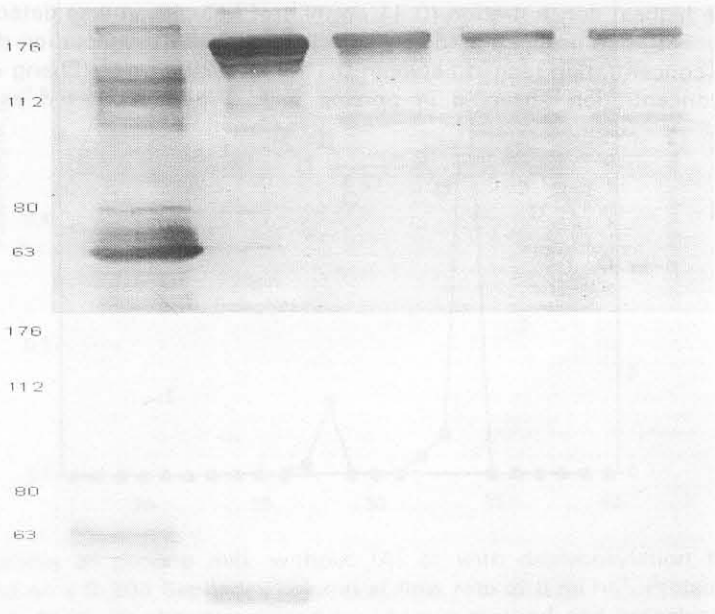

Fig. 2: Western blot analysis of betaglycan in milk samples collected at different days of lactation. Recombinant human betaglycan (1st lane from left) or porcine milk samples collected at day 1 (2nd lane from left), day 3 (3rd lane from left), day 7 (4th lane from left) or day 14 (5th lane from left) were electrophoresed on sodium dodecyl sulfate polyacrylamide gel under non-reducing (upper panel) or reducing condition (lower panel)

Characterization of Betaglycan in Milk: The molecular characteristics of betaglycan in porcine milk were initially determined by Western blot analysis (Fig. 2). Under non-reducing condition, the immunoreactive betaglycan in all samples appeared as a large compound of about 180 kDa (Fig. 2a). After pre-treatment of the samples with the reducing agent 2-mercaptoethanol, which breaks disulfide bonds in protein compounds, the immunoreactive betaglycan in the samples appeared as a much smaller compound of about 55 kDa (Fig. 2b). Under both non-reducing and reducing conditions, the immunoreactivity was highest in the day-1 milkand it declined progressively with the progress of lactation.

Incubation of milk samples with deglycosylation enzymes, chondroitinase ABC and heparitinase, released an immunoreactive betaglycan compound of about 110 kDa as shown in Wstern blot analysis under non-reducing condition (Fig. 3).

The molecular characteristics of betaglycan in porcine milk were further investigated by size-exclusion chromatography (Fig. 4). Porcine milk with or without deglycosylation was eluted on a column packed with Sephadex G-200 gel. Without deglycosylation, the immunoreactive betaglycan was eluted at the position greater than 200 kDa (Fig. 4a). However, after deglycosylation, the immunoreactive betaglycan peaked at the position of less than 150kDa (Fig. 4b). The immunoreactivity in the gel filtration elute was independent to the protein concentration (Fig. 4).

Discussion

In the present study soluble betaglycan was detected in all milk samples by both ELISA and Western blot analysis using a specific antibody. The results of both Western blot and size-exclusion chromatography revealed that the immunoreactive betaglycan in porcine milk is a large compound of 180-230 kDa (Fig. 2a; Fig. 4a). Digestion of milk samples with chronditinase ABC and heparitinase released an immunoreactive component of about 110-130 kDa (Fig. 3; Fig. 4b), suggesting that the betaglycan in porcine milk contains chondroitin sulfate and heparan sulfate glycosaminoglycan chains. These findings are similar to the reports of soluble betaglycan in cell culture medium (Andres *et al.*, 1989) and in rat milk (Zhang *et al.*, 2001). It has been reported that soluble betaglycan and betaglycan solubilized from cell membranes contain heparan sulfate and chondroitin fulfate glycosaminoglycan chains and a core protein of about 110 kDa (Andress *et al.*, 1989 and 1991). The present study also showed that the soluble betaglycan in porcine milk contains disulfide bonds, as pre-treatment of milk samples with a reducing agent 2-mercaptoethanol produced a small immunoreactive compound of about 55 kDa (Fig. 2b). This finding is consistent with the report of the solubilized membrane-anchored betaglycan that consists of two subunits of 95 kDa and 58 kDa respectively, linked by disulfide bonds (Philip *et al.*, 1999).

In the present study, the highest concentration (0.41 $\mu g\ ml^{-1}$) of betaglycan was detected in porcine colostrum (day-1 milk) and the concentration declined gradually to 0.25 $\mu g\ ml^{-1}$ by the lactation day of 14 (Table 1). In rat milk, soluble betaglycan concentration ranged between 0.15 and 0.29 $\mu g\ ml^{-1}$ (Zhang *et al.*, 2001), which was comparable with the concentration observed in porcine milk. When expressed as $\mu g\ g^{-1}$ milk protein, the

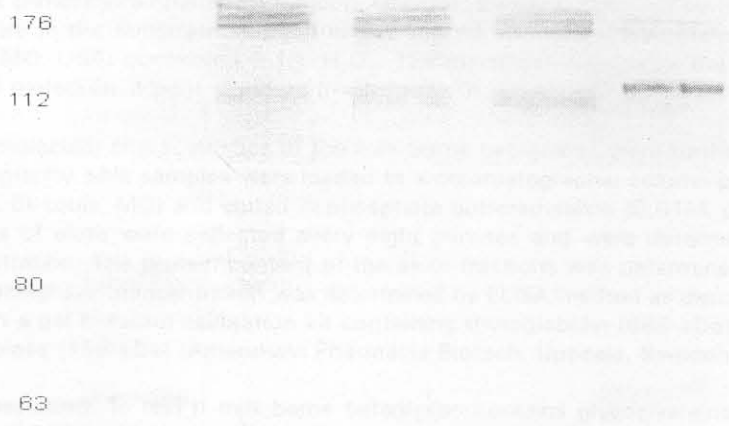

Fig. 3: Effects of enzymatic deglycosylation on the molecular profile of betaglycan in porcine milk determined by Western blot analysis. 1st lane from left, recombinant human betaglycan; 2nd lane from left, milk sample; 3rd lane from left milk sample digested with chronditinase ABC; 4th lane from left, milk sample digested with heparitinase; 5th lane from left, milk sample digested with both enzymes.

Table 1: Concentrations (mean ± SD) of protein and betaglycan in porcine milk collected at day 1, day 3, day 7 and day 14 of lactation.

Porcine milk	Protein (mg ml^{-1})	Betaglycan (ig ml^{-1})	Betaglycan/ protein (ig g^{-1})
Day 1	144.8 ± 15.1	0.41 ± 0.09	2.8 ± 0.5
Day 3	36.9 ± 1.6	0.30 ± 0.05	8.1 ± 1.0
Day 7	32.3 ± 5.1	0.27 ± 0.08	8.2 ± 1.1
Day 14	26.0 ± 3.7	0.25 ± 0.09	9.7 ± 3.7

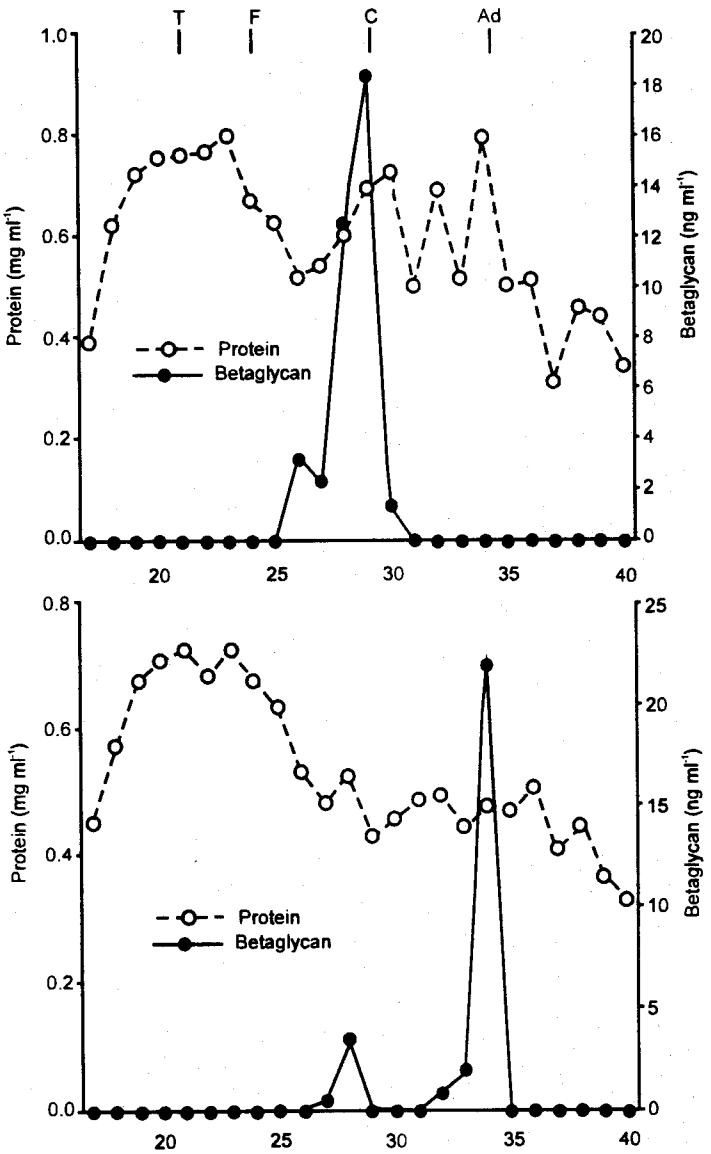

Fig. 4: Gel filtration profile of porcine milk without (A) or with deglycosylation (B). Milk samples were chromatographed on a G-200 Sephadex column at flow rate of 8 ml hr^{-1}. Protein concentration (dashed line) in the elute fractions was determined by Lowry's method and betaglycan level (solid line) was measured by enzyme-linked immunosorbent assay. The column was calibrated with thyroglobulin (T, 669-kDa), ferritin (F, 440-kDa), catalase (C, 232-kDa) and aldoase (Ad, 158-kDa)

concentration of betaglycan was significantly lower in porcine colostrum than in the mature milk (Table 1). The difference is likely due to a very high protein content in the colostrumand most of the proteins in porcine colostrum

are immuoglobulins (Xu, 2003).

The origin of the milk-borne betaglycan is unknown. It has been shown in humans that mammary epithelial cells express abundant membrane-anchored betaglycan (Sun and Chen, 1997). It has also been shown in rats that the lung membrane-anchored betaglycan is naturally shed off through protease cleavage at a site close to the transmembrane domain (Philip *et al.*, 1999). These findings suggest that milk-borne betaglycan may originate from ectodomain cleavage and shedding of the membrane-anchored betaglycan of the mammary epithelial cells. An alternative source, as suggested by Andres *et al.* (1989), is a direct cellular synthesis and secretion of soluble betaglycan. It has been reported that alternative mRNA splicing leads to co-expression of membrane-bound and secreted forms of various protein molecules (Gower *et al.*, 1988 and Giblin *et al.*, 1989).

The biological significance of the soluble betaglycan detected in porcine milk is unclear. It is shown in the present study that the milk-borne betaglycan is capable of binding TGF-β (Fig. 1). This finding together with our early observation of TGF-β in porcine milk (Xu *et al.*, 1999) suggests a possible physiological role of milk-borne betaglycan in modulating TGF-β activity. In a cell-free system, recombinant soluble betaglycan decreases the active form of TGF-β as measured by ELISAand in a in vitro cell culture system recombinant soluble betaglycan suppresses cellular responses to TGF-β (Nomura *et al.*, 2002). It has also been proposed that soluble betaglycan may be used to inhibit TGF-β-mediated tumor growth (Bandyopadhyay *et al.*, 2002). There is reported evidence showing that betaglycan inhibits TGF-β signaling by preventing type I and type II receptor complex formation (Eickelberg *et al.*, 2002). Betaglycan has also been found to be able to bind inhibin and has been suggested to play a role in inhibin-mediated reproductive function (MacConell *et al.*, 2002). The relevance of such function to the milk-borne betaglycan is unknown.

In summary, the present study has identified a soluble form of betaglycan in porcine milk and the compound is capable of binding TGF-β. These findings, together with early reports of TGF-β in porcine milk, suggest that betaglycan in porcine milk may play a role in modulating TGF-β activity in the gastrointestinal tract of the suckling young following oral ingestion.

Acknowledgements

This project was supported by a research grant from Hong Kong Research Grants Council (HKU7366/03M). The authors thank Mr. Victor Yeung for his technical assistance.

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