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Distribution and Developmental Changes of Transforming Growth Factor-beta Receptors in the Small Intestine of the Pig

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Abstract: Transforming growth factor-beta (TGF-β) has been detected in the milk of various species, including the pig. It has been speculated that milk-borne TGF-β may play an important role in postnatal adaptation of the gut in suckling animals. However, a prerequisite for such a role is the presence of functional TGF-β receptors in the gastrointestinal tract of the young. The aim of the present study was to determine if TGF-β receptors are present in the small intestine in newborn pigs. The study also aimed to examine the developmental changes of TGF-β receptors in the small intestine in suckling pigs. Immunohistochemical staining was used to localize the receptors in the tissue and Western blot analysis was used to determine their molecular characteristics. The level of the receptors present in the tissue was semi-quantified by a computerized image analysis and Western blot analysis. It was observed that TGF-β receptors I (RI), II (RII) and III (RIII) all were widely distributed along the small intestine in newborn unsuckled pigs, and the receptors were predominantly localized at the villus epithelium. There was a significant difference in the tissue level for different receptors in newborn piglets, and there was also a significant difference for the receptor level among different regions of the small intestine. The Brunner's glands in the duodenum of newborn piglets were strongly stained with RIII and the staining intensity in the glands reduced markedly following the onset of suckling. There was also a transient decline in the RI and RII density in the villus epithelium during the first day after birth but a marked increase in positively stained lymphocytes in the lamina propria of the intestinal villi during the immediate postnatal period. It was also observed that the staining of RI and RII at the villus epithelium shifted from the apical membrane in newborn and 3-day-old piglets to the basolateral membrane in 7- and 21-day-old pre-weaning piglets, while the staining intensity for RIII decreased markedly in 7-day-old pigs. These changes may correlate with the onset of endogenous TGF-β production. The findings strongly support the hypothesis of a regulatory role of milk-borne TGF-β in postnatal adaptation of the intestine in neonatal pigs.

Key words: TGF-β receptors, intestine, pig

Introduction

Transforming growth factor-beta (TGF-β) is a multifunctional polypeptide with profound biological effects in many developmental processes, including regulation of cell proliferation and differentiation, extracellular matrix formation, hematopoiesis, angiogenesis and inflammatory responses (Dignass *et al.*, 2001). In the gastrointestinal tract, TGF-β regulates enterocyte proliferation and differentiation (Booth *et al.*, 1995; van't Land *et al.*, 2002 and Berger *et al.*, 2003), promotes intestinal epithelial restitution after mucosal injury (Coerper *et al.*, 1997; Krishnamoorthy *et al.*, 2001; Mckaig *et al.*, 2002 and Henson, 2003), promotes intestinal epithelial barrier formation and epithelial integrity (Planchon *et al.*, 1999; Roche *et al.*, 2000 and Di Leo *et al.*, 2002) and modulates mucosa immune homeostasis (Penttila *et al.*, 1998; Luethviksson *et al.*, 2003 and Penttila *et al.*, 2003). TGF-β has been detected in the milk of various species, including the human (Saito *et al.*, 1993), cattle (Pakkanen, 1998), rat (Penttila *et al.*, 1998) and pig (Xu *et al.*, 1999). The concentration of TGF-β in milk is particularly high in colostrum and relatively low in late lactation. It has been speculated that milk-borne TGF-β may play an important role in postnatal adaptation of the gastrointestinal tract in suckling animals (Xu *et al.*, 2000). However, a prerequisite for any physiological effects of milk-borne TGF-β in suckling animals is the presence of functional TGF-β receptors in the mucosa of the gastrointestinal tract.

TGF-β exerts its biological actions through interactions with cell surface transmembrane receptor complex, that includes three components known as TGF-β receptor I (RI), II (RII) and III (RIII), respectively (Massague, 2000 and Liu, 2003). When TGF-β binds to the receptors, it acts as a dimeric assembly factor, bring RI and RII together. RII then activates RI, the latter subsequently phosphorylates intracellular SMAD proteins that regulate gene expression. RIII (also known as betaglycan) is a transmembrane proteoglycan that may not be directly involved in signal transduction but binds and presents TGF-β to the signaling RI and RII, and it may also function as a ligand reservoir (Lopez-Casillas, 1993). In a recent study, TGF-β receptors have been found in neonatal rat intestine (Zhang *et al.*, 1999). However there had been no similar studies reported in other species and no studies quantitatively evaluated

the developmental changes of TGF-β receptors in the small intestine following suckling. Therefore, the objectives of the present study were to determine whether TGF-β receptors exist in the gastrointestinal tract in newborn pigs, and if so, whether there are any changes in TGF-β receptor density and distribution associated with postnatal development. The study would provide supportive evidence for likely physiological effects of milk-borne TGF-β on the gastrointestinal development in the suckling young.

Materials and Methods

Tissue Sample Collection: Issue samples were collected from 24 Large White × Landrace pigs, including five newborn unsuckled piglets, five 1-day-old, five 3-day-old, five 7-day-old, and four 21-day-old suckling piglets. Prior to tissue sample collection, all experimental animals were first anaesthetized by peritoneal injection of pentobarbital sodium (Alfasan, Wberden, Holland) and then euthanased by intracardiac injection of an overdose of the drug. Immediately after death, the small intestine was removed and placed in chilled saline.

The small intestine was freed from the mesentery and divided at the junction of the duodenum and jejunum and at the junction of the ileum and caecum according to the earlier descriptions (Dyce *et al.*, 1987). The small intestine was then divided further into 4 equal lengths, designated proximal jejunum, distal jejunum, proximal ileum and distal ileum as in previous studies (McCance and Wilkinson, 1967; Widdowson *et al.*, 1976).

A block of tissue (about 1 cm in length) was taken from the middle of each region of the small intestine for histological studies. Intestinal mucosa was scraped off with a glass slide and immediately frozen in liquid nitrogen, and then stored at -70°C until further analysis of protein and DNA contents and Western blot analysis.

Immunohistochemical Examination: Tissue blocks collected for immunohistochemical study were immediately fixed in Bouin's fluid for 24 hours. The tissue blocks were then dehydrated through graded alcohol and embedded in paraffin wax. Cross tissue sections of 5 μm in thickness were dewaxed in xylene, rehydrated in alcohol solution of increased dilution, and then immersed in phosphate buffered saline (PBS). Endogenous peroxidase activity was eliminated by incubation with 1% H_2O_2 for 30 minutes, and nonspecific binding was blocked by incubation for 30 minutes with 2% normal goat serum (DAKO A/S, Denmark) or 2% bovine serum albumin (Sigma, MO, USA). The tissue sections were then incubated overnight at 4°C with polyclonal antibodies specifically against TGF-β receptor I, II and or III, respectively. After three washes in PBS, sections were further incubated for 2 hours at room temperature with biotin conjugated secondary antibodies. The primary and the secondary antibodies were obtained from a commercial supplier (Santa Cruz Biotech, CA, USA). After three washes in PBS, tissue sections were then incubated for 2 hours at room temperature with streptavidin-biotinylated horseradish peroxidase (Amersham, HK). Subsequently, the slides were developed in 3,3-diaminobenzidine solution (0.5 mg/ml) with the presence of 0.1% H_2O_2 for 10 minutes. After washing in distilled water, the slides were counterstained with hematoxylin (Sigma, MO, USA). The negative control sections were stained with the same procedures except the replacement of the primary antibodies with nonspecific rabbit IgG (DAKO, A/S, Denmark). The tissue sections were then examined under light microscope (Carl Zeiss Inc, Werk Göttingen, Germany), and positive staining was visualized as dark brown spots. The immunostaining intensity was semiquantified following the procedures described by Ruifrok *et al.* (1997a,b). Briefly, TGF-β receptors immunoreactivity was quantitated using a computerized image analysis system (Quantimet 500 image processing and analysis system, Leica Cambridge Ltd, Cambridge, England). This system consists of an IBM compatible computer with a image analysis software, a Leitz Wetzlar microscope (Germany), a color video camera (TK-1280E, JVC), and a 'live image' monitor. The image is converted to an analog electronic signal by the video camera and digitized by the computerized imaging board. Each point has a numeric location within the image. The software program calibrates the camera for white balance, optimizes the setting of the color bar, and automatically increases the threshold for maximal differentiation from positive and negative staining, until the size of the detected binary image accurately reflected TGF-β receptor positive tissue, seen both through the microscope and on the digitized image. Image artifacts, such as those produced by dirt and gut contents or folds in the tissue were eliminated from the analysis. The mean specific optical density, reflecting the immunostaining intensity, was calculated from the formula of OD = log10 [255/(255-grayscale value)] as described by Ruifrok *et al.* (1997b). The integrated OD, which is the product of the area stained and the mean specific OD, was taken as a semi-quantitative measure of the total amount of TGF-β receptor protein present.

To ensure objectivity of the method, a standardized procedure was established and used consistently throughout the study. At least 10 well-oriented villus-crypt units from three intestinal segments were made from each pig to obtain a single quantitative estimation. To test reproducibility, a slide was randomly selected and measured specific OD of RIII on five consecutive days. The difference between the OD values obtained on different days was less than 12%. To verify the reliability of the method, the specific OD of RIII staining was measured by two investigators, and the mean difference between the measurements of the two investigators was 12.8%.

Western Blot Analysis: Small intestine mucosa samples were defrosted and homogenized at 4°C in 50 mM Tris-HCl

buffer, pH 6.8, containing 5 mM EDTA, 10 μg/ml phenylmethylsulfonyl fluoride, and 0.2% NP-40 using a polytron homogenizer (Kinematica AG, Switzerland). Tissue debris was removed by centrifugation at 4500 rpm for 30 min. Protein concentrations of the tissue homogenates were measured with Lowry's method as described previously (Xu *et al.*, 1992).

Molecular characteristics of TGF-β receptors in the tissue homogenates were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as described by Rao and coworkers (Rao *et al.*, 2000). Briefly, tissue homogenates were diluted in the 50 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.05% bromophenol blue, and boiled for 5 min. The prepared samples of equal amounts of protein (50 μg) were then loaded to 12% (for RI and RII) or 7.5% (for RIII) SDS-PAGE gel and separated in an electrophoresis unit (Mini-Protean II Cell, Bio-Rad, CA, USA) at 120 V (constant voltage) for 1-2 h. Separated proteins were then transferred onto nitrocellulose membranes (Pharmacia Biotech, CA, USA) at 100 V/250 mA for 120 min at 4°C. The nitrocellulose membranes were subsequently blocked with Tris-buffered saline (100 mM Tris HCl, pH 7.5, 0.9% NaCl) containing 0.1% Tween 20 and 5% skimmed milk powder for 2 h at room temperature followed by overnight incubation at 4°C with polyclonal antibodies against TGF-β type I, II and or III receptors, respectively (Santa Cruz Biotech, CA, USA). The membranes were then washed with Tris-buffered saline, followed by further 2-hour incubation at room temperature with biotin conjugated goat anti rabbit IgG (Santa Cruz Biotech, CA, USA). After removing non-bound antibody, the membranes were then incubated for 2 hour in streptavidin-biotinylated horseradish peroxidase complex solution. Subsequently, the membranes were developed in 3,3-diaminobenzidine solution (0.2 mg/ml) in the presence of 0.1% H_2O_2 for 30 minutes. The membranes were air dried and stored in light-protected boxes at room temperature for further examination. The molecular weights of the immunoreactive bands were determined using a molecular weight standard (Invitrogen, CA, USA).

For quantitative analysis, the stained membranes were scanned and the intensity of the positive bands was measured using a Bio-Rad's image analysis systems (Bio Rad, CA, USA). The measured intensity of each sample was calibrated and converted to mg receptors per gram protein and μg receptors per μg DNA with the intensity of known amount of TGF-β receptor proteins (Santa Cruz Biotech, CA, USA).

Measurements of Mucosal Protein and DNA Contents: Sample of small intestinal mucosa were homogenized on ice with chilled saline using a polytron homogenizer (Kinematica AG, Switzerland). Protein concentration was then determined with the Lowry's method using bovine serum albumin as the standard. RNA and DNA were extracted from the tissue homogenates following the procedures described by Johnson and Chandler (1973). Briefly, duplicate 2ml the homogenates were mixed with 1 ml 0.6N perchloric acid. After standing in ice for 10 min, the precipitates were collected by centrifugation at 500 g for 10 min. The precipitates were washed twice with cold 0.2 N perchloric acid and suspended in 2 ml of 0.3 N KOH. They were then incubated at 37°C for 60 min. DNA and protein were precipitated by addition of 1 ml of 1.2 N perchloric acid. After standing in ice for 10 min the tubes were centrifuged at 500 g for 15 min. The DNA containing pellet was dissolved in 4 ml of 1 N perchloric acid by heating in a boiling water bath for 10 min. Denatured protein was removed by centrifugation at 500 g for 20 min. Duplicate 1 ml aliquots were assayed for DNA content using diphenylamine method (Giles *et al.*, 1964). Calf thymus DNA (Sigma, MO, USA) was used as the standard.

Statistics: The results were expressed as mean and standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to compare variances among various groups. Statistical differences between two groups were done by least significant difference (LSD) test. All analyses were conducted using SPSS for windows software version 11.0 (SPSS Inc. Chicago, USA). A p value of < 0.05 was considered significant.

Results

Distribution of TGF-β Receptors in the Small Intestine in Newborn Unsuckled Pigs: Immunohistochemical examination demonstrated that TGF-β receptors I, II and III were distributed along the small intestine in newborn unsuckled pigs. In the duodenum, staining of three receptors was seen on both apical and basal membranes of the villus epithelium. The staining of RI increased gradually along the villi towards villus tips (Fig. 1). The epithelial cells at the crypts were weakly stained with antibodies against RI and RII, but broadly stained with RIII. It is noteworthy that the entire epithelium of Brunner's glands was strongly stained with receptor III, but not with RI and RII (Fig. 2).

In the proximal jejunum and proximal ileum, RI and RII predominantly located on the basal side of the villus membranes. A faint staining of RI and RII at the crypts was also seen, while RIII were extensively expressed in the villi and the crypts (Fig. 1).

From the duodenum to the ileum, there are very few cells in the lamina propria that were positive stained in newborn unsuckled pigs. A few positively stained cells with RII and RIII were scattered in the Peyer's patches at the ileum region. The immunoreactivity with three TGF-β receptors was also noted in the wall of blood vessels

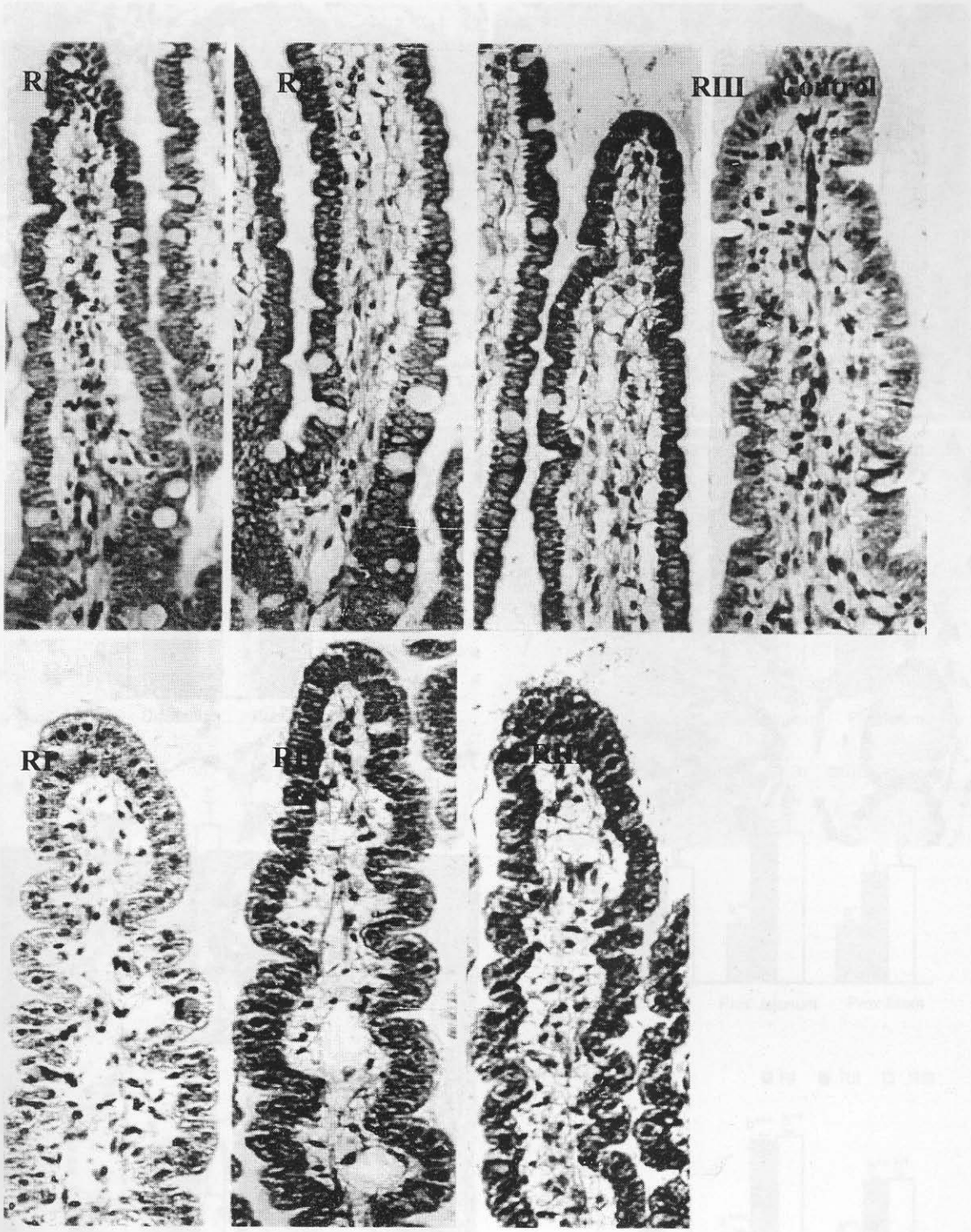

Fig. 1: Immunohistochemical microphotograph showing the expression of TGF-β receptortype I (RI), type receptor II (RII) and type receptor III (RIII) receptors in the duodenum (upper panel) and the proximal jejunum (lower panel) of newborn piglets (magnification 350×). Sections were incubated with commercial polyclonal antibodies to RI, RII and RIII, and color was developed using the avidin-peroxidase system described in materials and methods. No immunostaining was observed when the primary antibodies were replaced with nonspecific rabbit IgG (GControl)

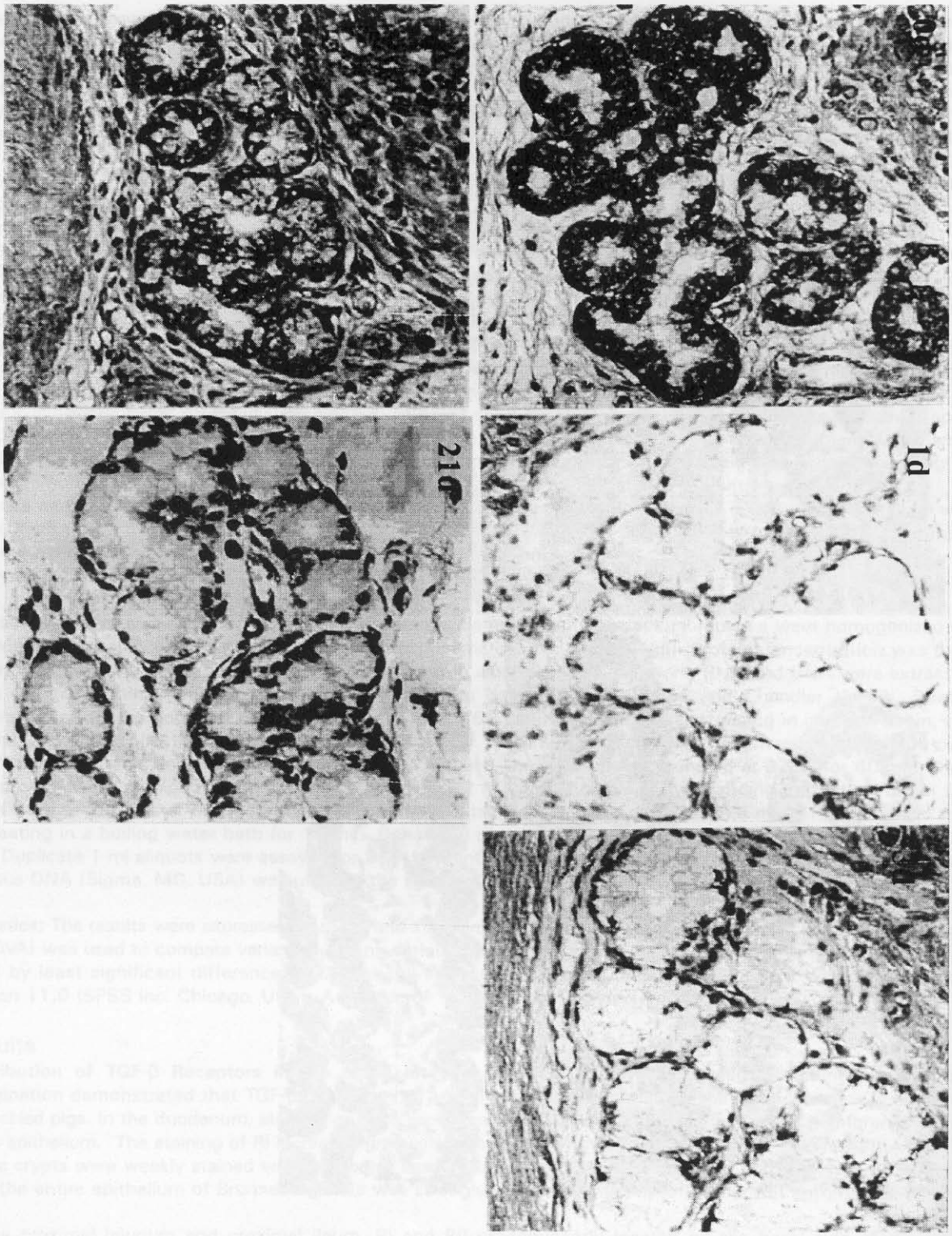

Fig. 2: Microphotograph of immunostaining of TGF-β type III receptor III in the Brunner's glands from newborn (0d), 1-d-old (1d), 3-d-old (3d), 7-d-old (7d) and 21-d-old (21d) piglets (magnification 350×).tissue sections were incubated with polyclonal antibody against RIII, and color was developed using the avidin-peroxidase system described in materials and methods. The Brunner's glands in newborn piglets (A) were strongly stained with RIII antibody, the staining faded away in 1- (B) and 3-day-old piglets (C) following the onset of suckling. In 7- (D) and 21-day-old piglets (E), the staining with RIII antibody regained intensity in the Brunner's glands

located in the submucosa, smooth muscle cells and Auerbach's plexus located between two muscle layers along the whole small intestine.

Western blot analysis confirmed the existence of TGF-β receptors I, II and III in the small intestinal mucosa in newborn pigs. Combined SDS-PAGE and Western blot analysis revealed that the molecular masses of receptor proteins for RI, RII and RIII were about 60, 70 and 80 kDa, respectively (Fig. 3).

The levels of RII and RIII estimated by Western blot analysis were generally higher than that of RI (Fig. 4A). The level of each receptor did not significantly differ among different regions of the small intestine in newborn pigs when estimated by Western blot analysis. When the levels of receptors were estimated by the Immunostaining, significantly differences were seen among different receptors as well as among different regions of the small intestine (Fig. 4B). The immunostaining intensity (OD) and the percentage area of positive staining on the intestinal villi for RII and RIII were significantly greater than those for RI along the whole small intestine. Among the different regions of the small intestine, the levels of receptor II and III estimated by the OD or by the percentage villus area of positive staining were highest in the proximal jejunum compared with those in the duodenum and those in the proximal ileum. Similar variation patterns were seen when the receptor levels were estimated as the integrated OD (Fig. 4B).

Developmental Changes of TGF-β Receptor Distribution in the Small Intestine: During the postnatal period and following the onset of suckling, there is a marked change in TGF-β receptor distribution in the small intestine. In the duodenal region, intensive staining with RI, RII and RIII antibodies was observed at the villus apical membrane, the staining intensity increased with animal age (Fig. 5). Intensive staining with RI antibody was also observed at the basal membrane of the villus epithelium of the duodenum in 21-day-old pigs. The Brunner's glands in newborn piglets were strongly stained with RIII antibody, the staining faded away in 1- and 3-day-old piglets following the onset of suckling (Fig. 2). In 7- and 21-day-old piglets, the staining with RIII antibody regained intensity in the Brunner's glands.

In the jejunum and ileum region of the small intestine, the villus epithelial cells were intensively stained with RI, RII and RIII antibodies in newborn piglets. There was a transient decline of the immunostaining in 1-day-old suckling piglets in association with obvious epithelial cell swelling (Fig. 6). There was also an apparent shift of the immunostaining from the apical membrane to the basal membrane of the epithelial cells in older animals. There were numerous positively stained cells scattered in the lamina propria of the intestinal villi. Quantitative analysis revealed that the number of the positively stained cells in the lamina propria increased progressively with animal age (Fig. 7).

Quantification of immunohistochemical staining in the duodenal villi by image analysis showed that the staining intensity (OD) of RI and RII was relatively constant among animals of different ages, while the positive staining areas for all three receptors significantly increased with age (Fig. 8B). The OD of RIII slightly increased at d1 and d7, and significantly reduced in 21-day-old pre-weaning pigs, compared with that in 7-day-old pigs (Fig. 8B). Western blot analysis also revealed a similar decline of RIII expression in the duodenal mucosa at d21 (Fig. 8A). On the other hand, quantification of immunohistochemical staining at the Brunner's glands showed a distinctive immunoactivity change of RIII during the neonatal period (Fig. 9).

In the jejunum, both the staining intensity (OD) and the area of positive staining for all three receptors declined significantly during the first day, and then returned to the birth values by the third day. A significant increase in the OD and the area of positive staining area for RI was noted at d21 (Fig. 10B). In contrast, the OD of RIII decreased significantly by day d7 (Figure 10B). Western blot analysis also showed that the level of RI (mg/g protein and g/g DNA) was significantly higher in 21-day-old pigs, compared with that in 7-day-old pigs, while RIII level (g/g DNA) significantly decreased at d7 (Fig. 10A).

In the proximal ileum, similar to that in the jejunum, there was a transient decline in the staining intensity and the area of positive staining for all three receptors at the villus regions during the first three days of life, and then returned to the birth value by d7. A significant increase of positive staining area for RI was found at d21 (Figure 11B). Similarly, Western blot analysis showed that the level of RI (μg/g mucosa) significantly increased in 21-day-old pigs, compared with that in 7-day-old pigs, and there was a transient decrease of RIII (μg/g mucosa) at d7 (Fig. 11A).

Discussion

Immunohistochemical examination in the present study revealed that TGF-β receptors were distributed along the small intestine in newborn piglets. In the small intestine, three TGF-β receptors were expressed predominantly at the villus epithelium, and the epithelial cells at the crypts were only weakly stained with RI and RII antibodies. Similar distribution pattern has also been seen in the small intestine of neonatal rats (Winesett *et al.*, 1996 and Penttila *et al.*, 1998), mice (Zhang *et al.*, 1997) and human infants (Chung *et al.*, 2002). In the postnatal rat small intestine, TGF-β receptor I was expressed by the epithelial cells of the villi, and the staining on the apical membrane

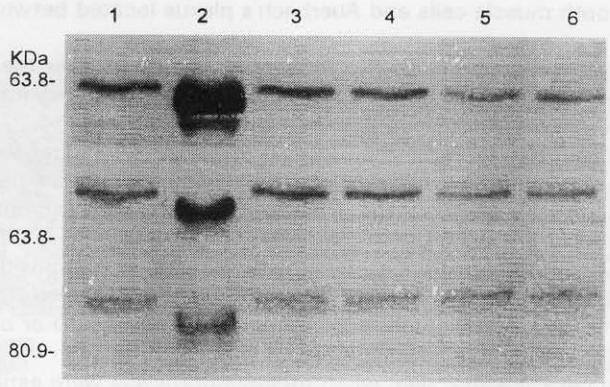

Fig. 3: Western blot analysis of TGF-β type receptor I (RI), type receptor II (RII) and type receptor III (RIII) receptors in the small intestinal mucosa of newborn piglets. Lane 1: duodenum; Lane 2: TGF-β receptor protein (Santa Cruz Biotech, CA, USA); Lane 3: proximal jejunum; Lane 4: distal jejunum; Lane 5: Proximal ileum; Lane 6: distal ileum. The intestinal mucosa homogenates (50 μg protein) were separated by electrophoresis using 12% (RI and RII) or 7.5% (RIII) SDS-PAGE gel, transferred to a nitrocellulose filter and subjected to Western blot analysis with RI, RII or RIII antibodies.

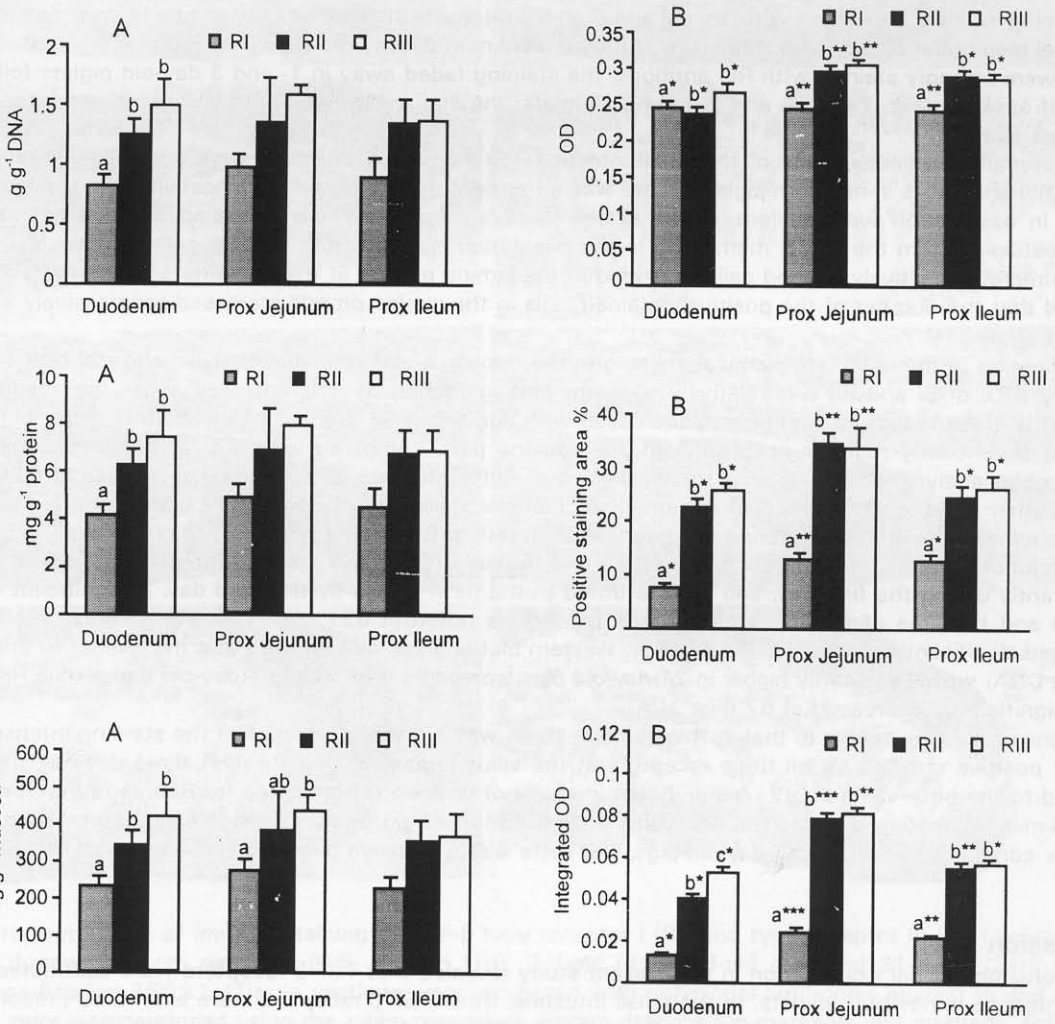

Fig. 4: The levels of TGF-β type receptor I (RI), type receptor II (RII) and type receptor III (RIII) receptors in the small intestine in newborn pigs estimated by Western blot analysis (A) or by immunostaining (B). The results were expressed as the mean (histogram) and the standard error of the mean (bar over the top of the histogram). Histograms marked with different letters (a, b or c) indicate significant differences among different receptors and histograms marked with different numbers of stars (*, ** or ***) indicate significant differences among different regions of the small intestine

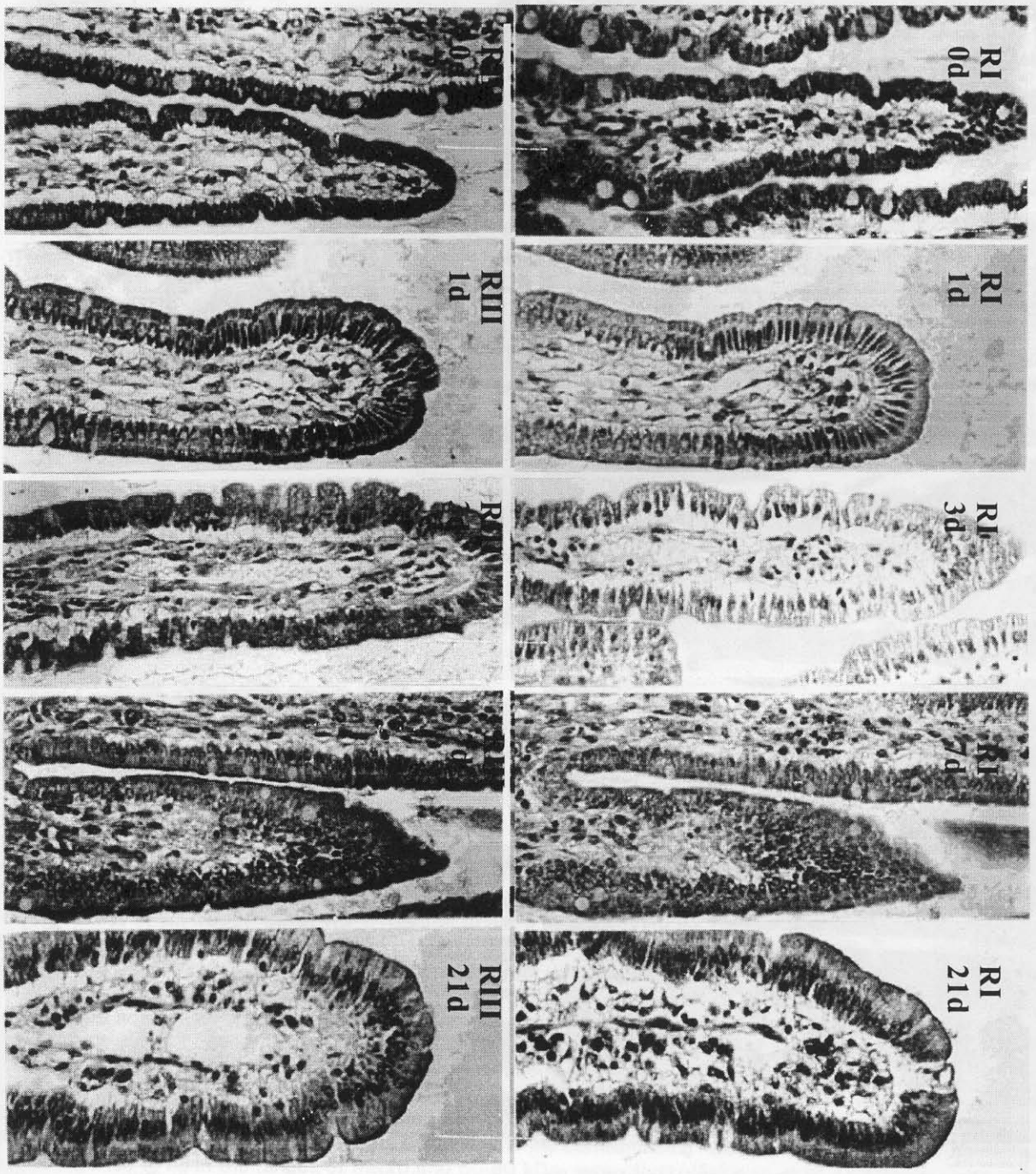

Fig. 5: Microphotograph of immunostaining of TGF-β type receptor I (RI) and type receptor III (RIII) receptors in the duodenum from newborn (0d), 1-d-old (1d), 3-d-old (3d), 7-d-old (7d) and 21-d-old (21d) piglets (magnification 350×). tTissue sections were incubated with polyclonal antibodies against RI and or RIII, and color was developed using the avidin-peroxidase system described in materials and methods. Following the onset of suckling, lintensive staining with RI and RIII antibodies was observed at the villus apical membrane in newborn piglets, the staining intensity increased with animal age. Intensive staining with RI antibody was also observed at the basal membrane of the villus epithelium in newborn piglets in 21-day-old pigs.

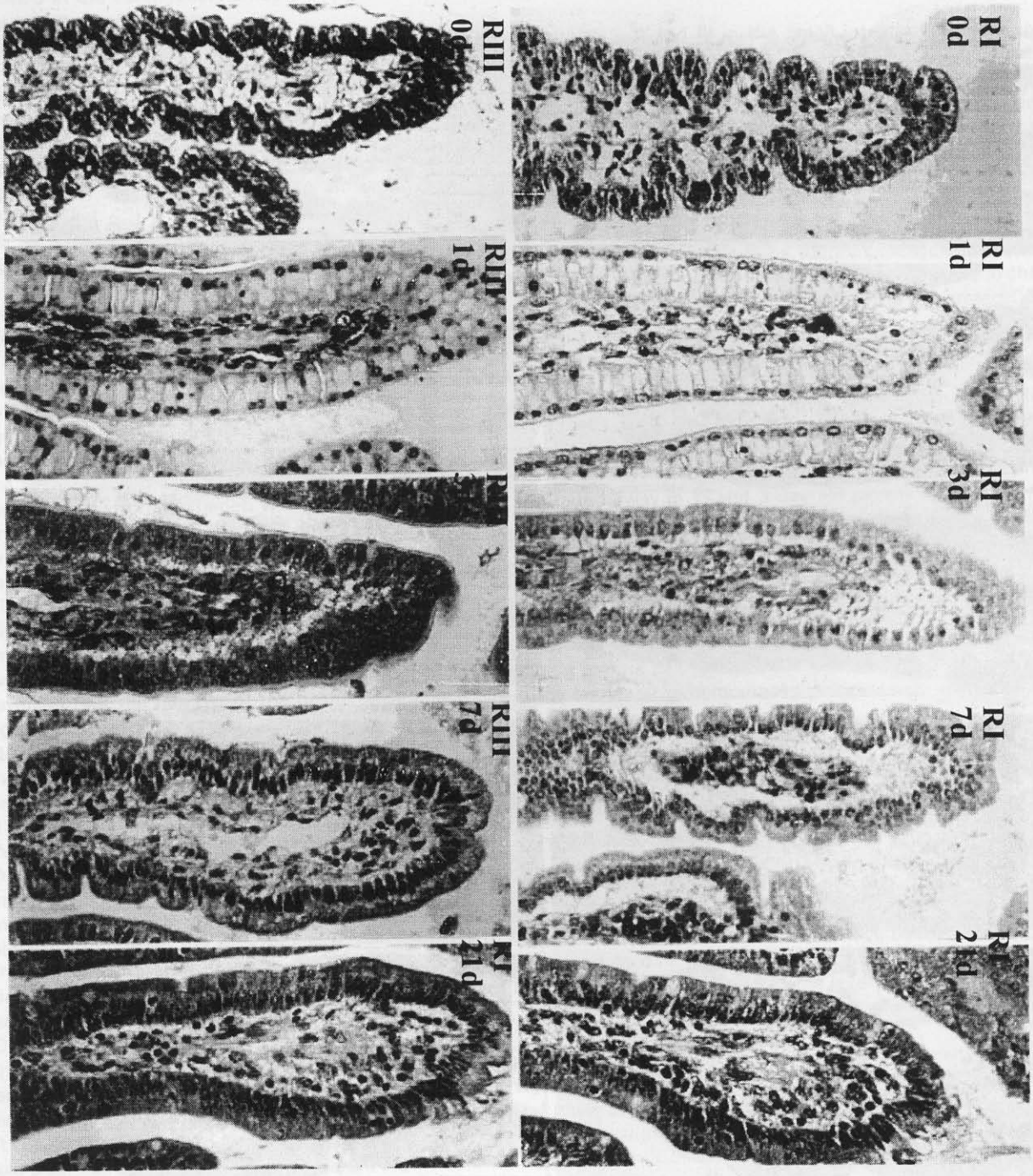

Fig. 6: Microphotograph of immunostaining of TGF-β type receptor I (RI) and type receptor III (RIII) receptors in the proximal jejunum from newborn (0d), 1-d-old (1d), 3-d-old (3d), 7-d-old (7d) and 21-d-old (21d) piglets (magnification 350 ×). Tissue sections were incubated with polyclonal antibodies against RI and or RIII, and color was developed using the avidin-peroxidase system described in materials and methods. The villus epithelial cells were intensively stained with RI and RIII antibodies in newborn piglets. There was a transient decline of the immunostaining in 1-day-old suckling piglets in association with obvious epithelial cell swelling. There was also an apparent shift of the immunostaining from the apical membrane to the basal membrane of the epithelial cells in older animals. There were numerous positively stained cells scattered in the lamina propria of the intestinal villi

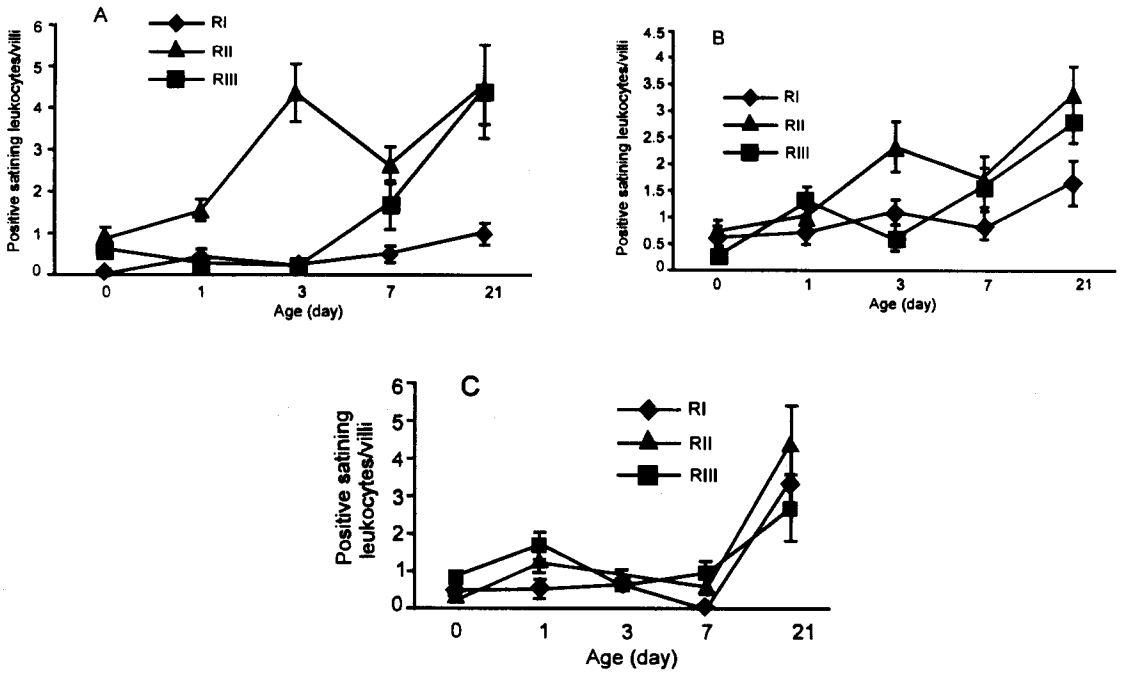

Fig. 7: The number of positively stained cells with TGF-β type receptor I (RI), type receptor II (RII) and type receptor III (RIII) receptors in the villus lamina propria in the duodenum (A), proximal jejunum (B) and proximal ileum (C) in piglets of different age. Results were expressed as the mean and the standard error of the mean

increased with age (Zhang *et al.*, 1999). In the human infants, TGF-β receptors were expressed on both apical and basolateral membranes of the small intestinal villus epithelium, and the staining was more prominent in differentiated villus tips (Chung *et al.*, 2002). It is well known that TGF-β inhibits intestinal epithelial cell proliferation but stimulates epithelial cell differentiation (Booth *et al.*, 1995; Murphy *et al.*, 1998; van't Land *et al.*, 2002 and Berger *et al.*, 2003). The predominant expression of TGF-β receptor I and II on the villus tips may imply that TGF-β functions to arrest growth of cells emerging from the crypt and plays a role in maintaining the epithelial cell homeostasis along the crypt-villus axis. In contrast to the distribution of TGF-β receptor I and II at the villi, TGF-β receptor III was extensively expressed in the villi and the crypts. TGF-β receptor III is a transmembrane proteoglycan with a biological function of binding and presenting the ligand to the signaling receptors I and II (Lopez-Casillas et al., 1993). Since TGF-β receptor III has a higher affinity for TGF-β2, extensive expression of TGF-β receptor III in the intestine of newborn pigs may reflect a physiologic necessity for milk-derived TGF-β2, the predominant isoform in porcine milk, to access signaling receptors in the gut. Coexistence of three TGF-β receptors in the small intestine epithelium in the newborn pigs would allow milk-derived TGF-β, especially TGF-β2, to exert its biological actions via interaction with the ligand-binding RIII and signaling RI and RII.

Western blot analysis confirmed the existence of TGF-β receptors in the gastrointestinal mucosa in newborn piglets. The analysis revealed apparent molecular masses of about 60, 70 and 80 kDa for TGF-β receptor I, II and III, respectively. The estimated molecular masses of receptor I and II are comparable with the values reported in the literature. The molecular masses of receptors I and II isolated form cultured pig Leydig cells were reported to be 53 and 80 kDa respectively (Goddard *et al.*, 2000), while the corresponding values for human TGF-β receptor I and II were reported to be in the ranges of 55-70 and 60-80 kDa, respectively (Eskinazi *et al.*, 1998 and Hatthachote *et al.*, 1998). TGF-β receptor III consists of glycosaminoglycan chains and a core protein of 853 amino acid residues (Philip *et al.*, 1999). The core protein contains two ectodomains being held together by disulfide bonds. Using an isotope labeled ligand-binding assay, the molecular mass of TGF-β receptor III isolated from cultured pig Leydig cells was estimated to be around 300 kDa (Goddard *et al.*, 2000). In the present study, the molecular mass of the receptors was estimated by SDS-PAGE under reducing condition that dissociates disulfide linkages. Therefore, the receptor III of about 80 kDa detected in the present study is likely to be a fragment of the intact receptor.

The semiquntitative immunohistaining method for detection of a specific protein used in this study is well established and has been used intensively (Ruifrok *et al.*, 1997a b, 2001and 2003). The measurement with image

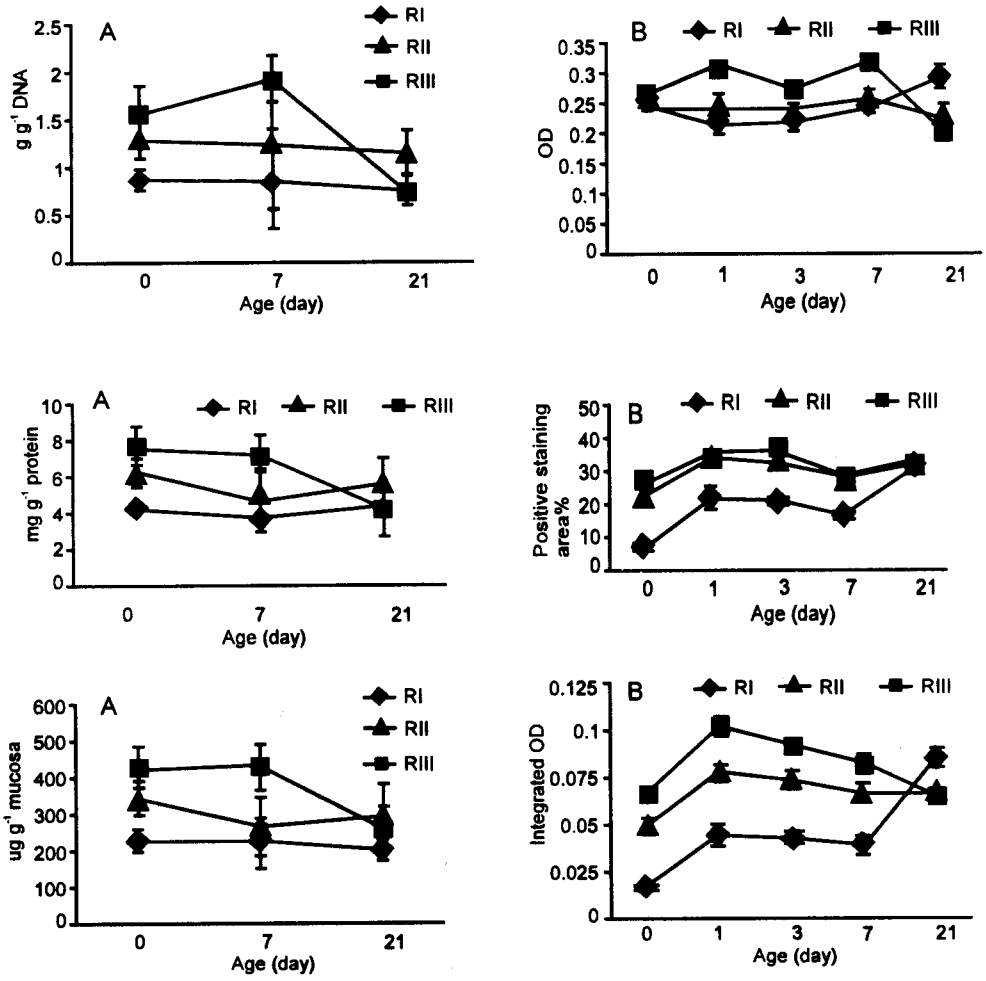

Fig. 8: The levels of TGF-β type receptor I (RI), type receptor II (RII) and type receptor III (RIII) receptors contents in the duodenum of piglets of piglets of different age estimated by Western blot analysis (A) or by immunostaining image analysis (B). Results were expressed as the mean and the standard error of the mean.

analysis is objective because it uses full automated threshold settings, and the measurement is reproducible, as shown by the relatively small errors and reproducible results in repeated measurements. There are at least two reasons to think that the differences in the patterns of expression of TGF-β receptors and probably not due to differences in the affinities of the antibodies used. First, the intensity of immunostaining for TGF-β receptors is equivalent at certain stages of development, suggesting that the different intensities of staining for the TGF-β receptors reflect real differences in the presence or availability of the antigens. Second, western blot analysis shows detection of similar amount of receptors by their respective antibodies.

The results of the immunostaining revealed that the three types of TGF-β receptors co-expressed in the small intestine in newborn unsuckled pigs. The level of expression differed along the small intestine. The levels of three types of TGF-β receptors were highest in the jejunum. Among the receptors, the immunostaining intensity of RI was significantly lower than that of RII and RIII. Mariano *et al.* (1998) reported that expression of RI was generally higher than that of RII in some tissues of the mouse embryo at the beginning of organogenesis, but the level of TGF-β RII increased dramatically during late organogenesis. The different degree expression for different types receptors in the small intestine in newborn pigs may allow more precise control of signal transduction during development. Alternatively, different degree expression may suggest a differential contribution of the receptors to the diverse biological actions of TGF-β. Previous studies have suggested TGF-β receptor I may mediate extracellular matrix deposition, and RII may mediate growth inhibition (Chen *et al.*, 1993 and Miettinen *et al.*, 1994).

It has also been observed in the present study that the duodenal Brunner's glands were intensively stained with RIII antibody in newborn piglets. The staining intensity in the glands reduced markedly in 1- and 3-day-old piglets

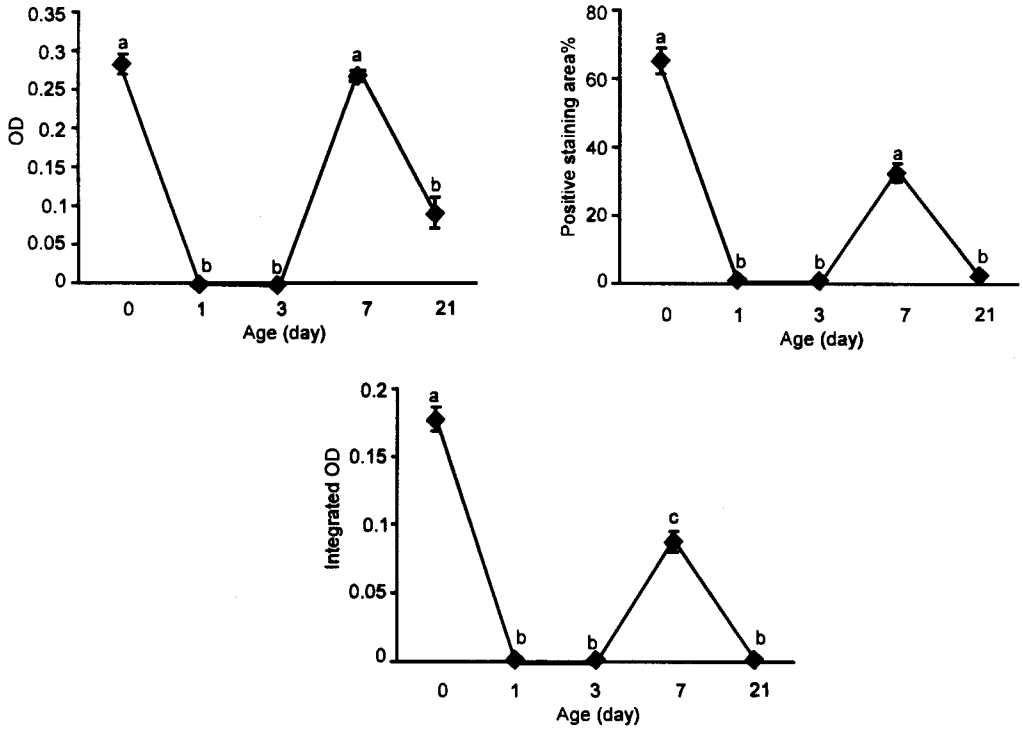

Fig. 9: The staining intensity (OD), the percentage area of positive staining and the semi-quantified protein level (integrated OD) of TGF-β-type receptor III (RIII) receptor in the duodenal Brunner's glands of piglets of different age. Results were expressed as the mean and the standard error of the mean

following the onset of suckling. On the other hand, the staining of RIII on the apical side of intestinal villus epithelium increased following suckling. These findings suggest that TGF-β receptor III are produced in these excretory glands and are secreted into the gastrointestinal lumen. The increment of RIII staining at the duodenal villi following suckling may be due to exogenous supplementation of RIII from maternal milk or due to endogenous excretion by the Brunner's glands. It has been reported that the gut of the rat pups fed on maternal milk contains more TGF-β RIII when compared with the pups fed on milk-replacer (Zhang *et al.*, 2001). TGF-β receptor III is a transmembrane proteoglycan with a biological function of binding and presenting the ligand to the signaling receptors I and II (Lopez-Casillas *et al.*, 1993). The ectodomain of the receptor can be effectively shed from the cell surface membrane and be released into the body fluid (Philip *et al.*, 1999). Soluble TGF-β receptor III has been detected in various biological fluids, including cell culture medium, blood serum and extracellular fluid (Andres *et al.*, 1989). More recently, soluble TGF-β receptor III has been detected by Zhang and coworkers (Zhang *et al.*, 2001) in rat milk and by our own laboratory in porcine and human milk (Cheung *et al.*, 2002). It has been reported that soluble TGF-β receptor III binds TGF-β independent of other receptors and competitively inhibits the binding of TGF-β to the cell surface receptors (Lopez-Casillas *et al.*, 1994). From the above discussion, it may be speculated that TGF-β receptor III secreted by the Brunner's glands and those presented in the maternal milk may function as a TGF-β carrier, transfer and present the ligand to appropriate tissues.

It was observed in the present study that the staining of RI and RII at the intestinal epithelium shifted from the apical membrane in 3-day-old piglets to the basolateral membrane in 7- and 21-day-old pre-weaning pigs. These changes may correspond to the shift of exogenous milk-borne TGF-β to endogenous production of TGF-β by the intestinal tissue. The concentration of TGF-β is high in porcine colostrum and it declines rapidly during the first three days of lactation (Xu *et al.*, 1999). The reciprocal changes in milk TGF-β concentration and intestinal endogenous production of TGF-β has been reported in rats (Penttila *et al.*, 1998). Our previous study also showed that the staining for TGF-β1 in the pig small intestine did not appear before day 7 and increased markedly in 21-day-old pigs (Mei *et al.*, unpublished data). Increase of TGF-β receptor I and II on the apical membrane of intestinal epithelium during the first three days may play a role in maintaining the mucosa barrier and integrity (Di Leo *et al.*, 2002), and therefore limit antigen exposure in the periphery (Planchon *et al.*, 1999; Roche *et al.*, 2000 and Planchon *et al.*, 1994 and 1999). TGF-β has been shown to reduce the capacity of interferon-γ or the human pathogen *Cryptosporidium parvum* to disrupt epithelial cell barrier function (Planchon *et al.*, 1999; Roche *et al.*,

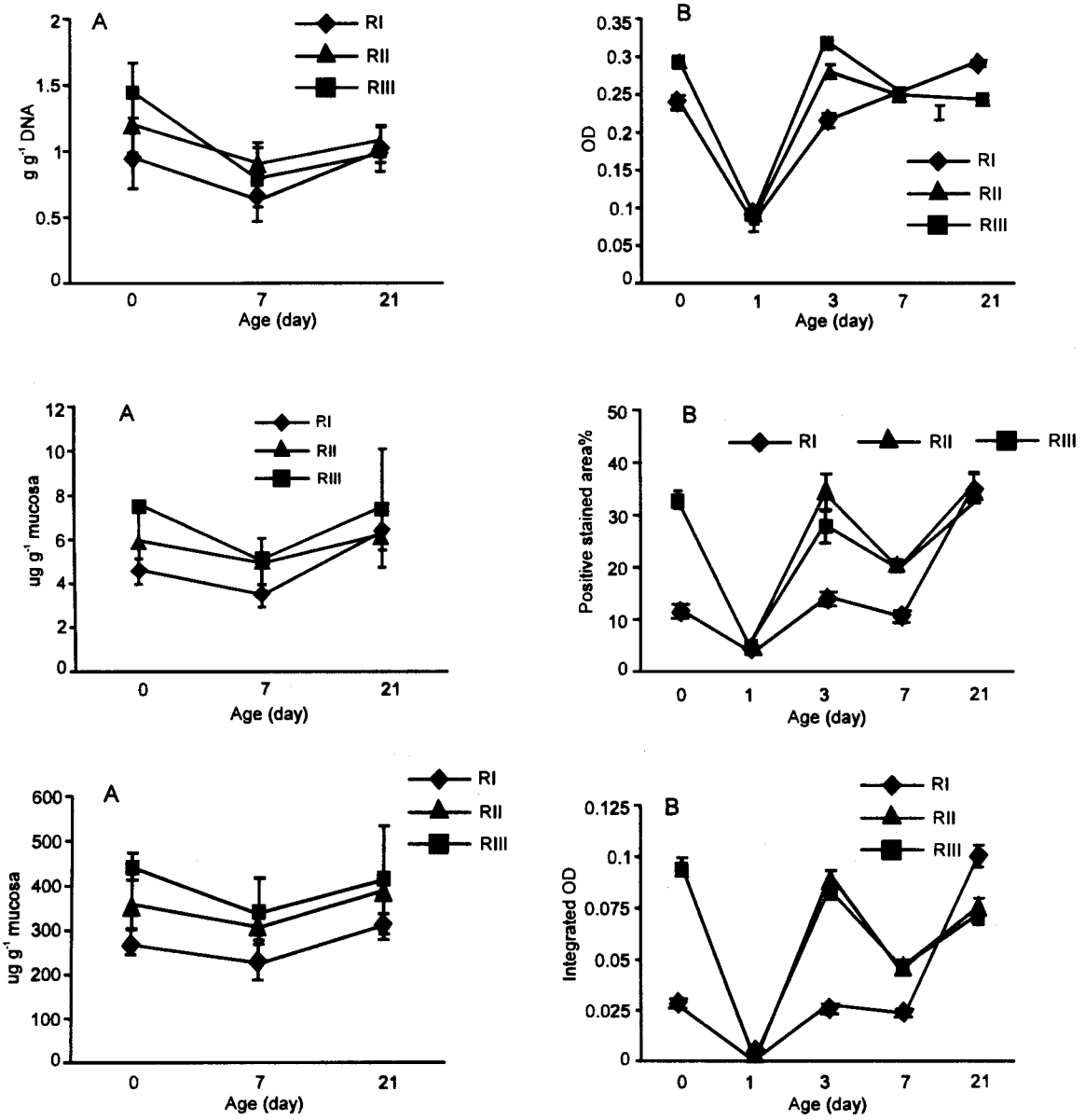

Fig. 10: TGF-β receptor I (RI), receptor II (RII) and receptor III (RIII) contents in the proximal jejunum of piglets of different ageThe levels of TGF-β type I (RI), type II (RII) and type III (RIII) receptors in the proximal jejunum of piglets of different age estimated by Western blot analysis (A) or by immunostaining image analysis (B). Results were expressed as the mean and the standard error of the mean.

2000 and Di *et al.*, 2002).

It was also revealed that the level of RIII in the proximal jejunum and ileum was low in 7-day-old pigs. Similar results have been shown in rats (Zhang *et al.*, 1999). In the rat small intestine, RIII was distributed throughout the mucosa at early age, but diminished from the epithelium postweaning (Zhang *et al.*, 1999). Andres *et al.* (1991) also reported that RIII was less abundant in adult tissues compared with fetal tissues. As an accessory receptor, RIII binds and presents TGF-β to RII. RIII binds all three TGF-β isoforms but has a higher affinity for TGF-β2 than for TGF-β1 or TGF-β3 (Cheifetz *et al.*, 1987). High expression level of RIII in the suckling pig intestine could allow milk-derived TGF-β, especially TGF-β2 to be presented and bind to RII and RI signal receptors and exerts its physiological effect in the small intestine. Reduction of expression of RIII during this period may correlate with the onset of endogenous TGF-β1 production by enterocytes. Unlike TGF-β2, β1 can directly interact with RII, which

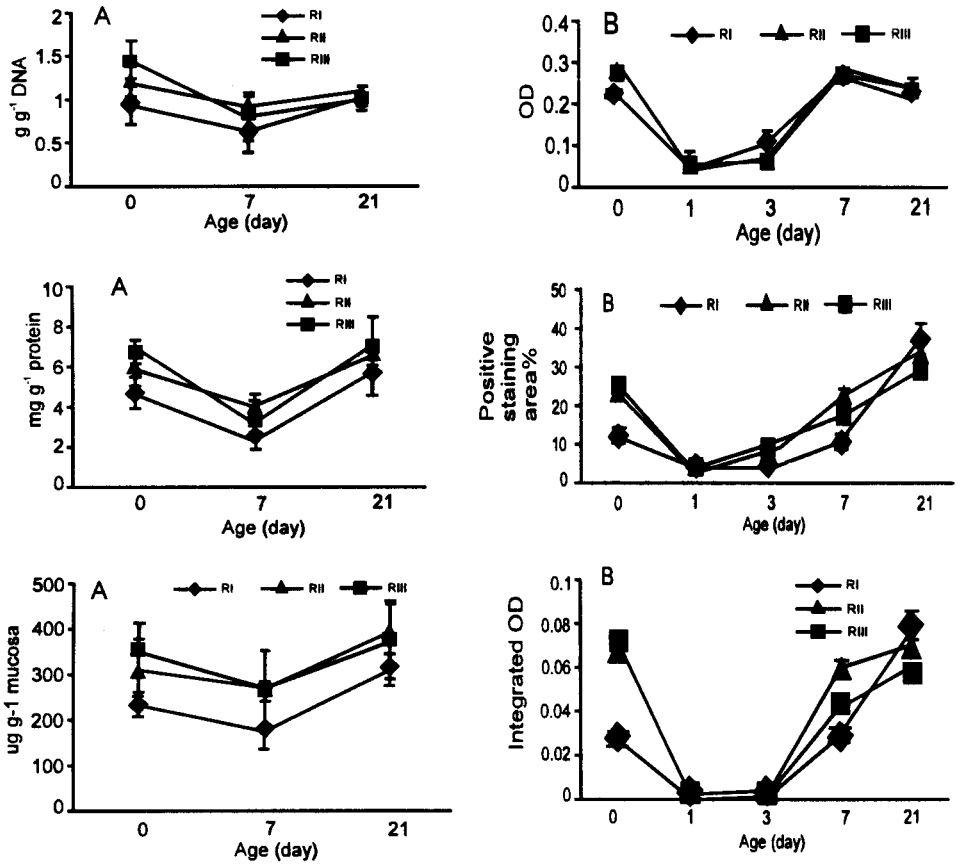

Fig. 11: TGF-β receptor I (RI), receptor II (RII) and receptor III (RIII) contents in the proximal ileum of piglets of different ageThe levels of TGF-β type I (RI), type II (RII) and type III (RIII) receptors in the proximal ileum of piglets of different age estimated by Western blot analysis (A) or by immunostaining image analysis (B). Results were expressed as the mean and the standard error of the mean.

then leads to signal transduction in conjunction with RI. With decline of milk-derived TGF-β2, and increase of endogenous production of TGF-β1, the need for RIII to bind and present ligand decreased.

Quantitative analysis of the receptor staining intensity and the area of positive staining of the intestinal villi showed a marked decline during the first day after birth in the proximal jejunum and ileum, suggesting a reduction in the level of TGF-β receptors present in the villi during this period. Such transient decrease in TGF-β receptors in the villi may be a secondary response to the high concentration of TGF-β in the colostrum. The reduced TGF-β receptors at the first day after birth may play a role in triggering a proliferative response in the crypts by reducing growth inhabiting signals from the villi. Receptor down-regulation following exposure to its ligand is a common phenomenon. Such phenomenon has been reported for epidermal growth factor receptors (Canesi *et al.*, 2000), somatostatin receptors (Koenig *et al.*, 1997) and opioid receptors (Chakrabarti *et al.*, 1997). Alternatively, the transient decline of TGF-β receptor I and II may relate to the ingestion of milk-borne polyamines. It has been reported that maternal milk contains a high level of polyamines (Loser, 2000) and polyamine inhibits TGF-β receptor expression in intestinal epithelial cell line (Rao *et al.*, 2000).

It was also observed in the present study that the number of scattered cells positively stained with antibodies against three TGF-β receptors increased markedly in the intestinal lamina propria in neonatal pigs following the onset of suckling. These positively stained cells are most likely immunological lymphocytes. It has been shown in neonatal rats that immune cells, including T cells, B cells and veiled dendritic cells in the lamina propria expressed TGF-β receptor III (Zhang *et al.*, 1999). This increment may correspond to the postnatal maturation of mucosal immune system. Exposure of mucosal immune system to food and bacterial antigens leads to a population expansion of T and B cells in the gastrointestinal mucosa and to an increase in the number, size and germinal centers of Peyer's patches (Cummins and Thompson, 1997). It is known that TGF-β is a potent regulator in the mucosal immune system and plays a crucial role in maintaining mucosal immune homeostasis (Letterio and Roberts,

1998; Murphy, 1998; Luethviksson *et al.*, 2003 and Penttila *et al.*, 2003). TGF-β null mice remained physiologically normal while on maternal milk but developed a degenerative syndrome characterized by weight loss and infiltration of inflammatory cells into the heart, lungs and salivary glands soon after weaning (Christ *et al.*, 1994). In human infants, TGF-β derived from maternal milk appears to regulate gut mucosal immune system and prevents the development of atopic disease or food allergy (Kalliomaki *et al.*, 1999; Saarinen *et al.*, 1999 and Chung *et al.*, 2002). The major effects of TGF-β on the mucosal immune system seem to be regulation of IgA production (Weiner, 2001), suppression of proinflammatory cytokine responses (Hahm *et al.*, 2001; Fiocchi, 2001; Strober *et al.*, 2001 and Claud *et al.*, 2003) and induction of oral tolerance (Weiner, 2001 and Penttila *et al.*, 2001). Loss of TGF-β signaling in the intestine contributed to intestinal inflammation (Hahm *et al.*, 2001). In oral toleranced mice TGF-β-secreting regulatory cells in Peyer's patches was significantly increased (Neurath *et al.*, 1996 and Weiner, 2001). Loss of TGF-β signaling in the intestine contributed to intestinal inflammation (Hahm *et al.*, 2001; Fiocchi, 2001 and Strober *et al.*, 2001). In oral toleranced mice TGF-β-secreting regulatory cells in Peyer's patches was significantly increased (Neurath *et al.*, 1996 and Weiner, 2001).

In summary, the present study demonstrated for the first time the existence of TGF-β receptors in the small intestine in newborn pigs. This study also showed distinctive changes of TGF-β receptor density and distribution in the small intestine following the onset of suckling, suggesting that expression of receptors is regulated in a spatio-temporal manner in the small intestine during the postnatal adaptation and development period. The changes are most likely related to the exposure to milk borne TGF-β ligands. These findings strongly support the hypothesis that milk-borne TGF-β plays an important role in postnatal adaptation of the gastrointestinal tract in neonatal animals.

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