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Vitamin D Receptor Distribution in Intestines of Domesticated Sheep *Ovis ammon* f. aries

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Abbreviated title: Vitamin D Receptors in Sheep Intestine

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ABSTRACT: The biologically active form of vitamin D, i.e., 1,25-dihydroxycholecalciferol or calcitriol, plays an important role in bone metabolism and calcium homeostasis, which is often disturbed at the onset of lactation in high milk-yielding domestic ruminants. Gene transcription is modulated via vitamin D receptors, but nongenomic effects of vitamin D via membrane receptors have also been described. In the intestines vitamin D promotes calcium absorption via vitamin D receptors. Vitamin D receptors are of clinical relevance, but have not been systematically assessed within all segments of the intestine in any species. Thus, we present for the first time an immunohistochemical study of the distribution patterns of the vitamin D receptor protein in sheep, which may be the basis for present and future investigations on mineral homeostasis in domestic ruminants.

Tissue probes of the intestines were collected from 5 lambs and 5 nonlactating and nonpregnant dams, fixed in formalin, embedded in paraffin and used for the assessment of vitamin D receptor protein. Nuclear vitamin D receptor immunoreaction was scored semiquantitatively and exhibited a segment specific distribution pattern. Goblet cells always were devoid of any vitamin D receptor immunoreaction. Surface epithelial cells and enterocytes of the crypt openings generally demonstrated only a weak immunoreaction. Basally and/or intermediately located crypt epithelial cells exhibited stronger immunoreactions in duodenum, jejunum and colon descendens. This basal/intermediate to superficial gradient was most pronounced in the duodenum and less evident in jejunum and colon descendens and not observed in ileum and cecum. There were no age-dependent variations in vitamin D receptor protein expression.

Results demonstrate that intestinal vitamin D receptor distribution patterns are segment-specific and strongest immunoreactions correlate with highest intestinal calcium absorptive activities as reported in literature. Strong expression of vitamin D

receptors within the lower half of crypts also suggests a role for calcitriol in epithelial differentiation and cellular homeostasis.

Vitamin D is a secosteroid prohormone, which is obtained from the diet and by the action of sunlight on the skin via conversion of 7-dehydrocholesterol to previtamin D. It exerts its effects through its active form 1,25-dihydroxycholecalciferol or calcitriol, which is activated from the precursor molecule by 25-hydroxylase in the liver and the 1α-hydroxylase in the kidneys. Calcitriol circulates in blood bound to the vitamin D binding protein. Within target cells calcitriol is bound to a binding protein. Subsequently it interacts with the vitamin D receptor and induces heterodimerization of the calcitriol-vitamin D receptor complex with retinoic X receptor. The heterodimer binds to response elements on target genes. Coregulators are recruited to link the dimer to the transcriptional machinery and thereby modulate gene transcription (Horst et al., 1994; Duque et al., 2002; Christakos et al., 2003; Holick, 2003; Evans et al., 2004; Fleet, 2004; Mizwicki et al., 2004; Dusso et al., 2005; Horst et al., 2005; Hendy et al., 2006; Yamagishi et al., 2006; Zella et al., 2006; Schröder and Breves, 2007).

A number of other pathways involving rapid nongenomic effects have also been described in addition to the pathway of action described here. These include interaction of calcitriol with a membrane receptor, either a novel receptor or the known vitamin D receptor, thereby activating cell signalling pathways (Nemere and Campbell, 2000; Nemere et al., 2000; Holick, 2003; Evans et al., 2004; Fleet, 2004; Mizwicki et al., 2004; Dusso et al., 2005; Hendy et al., 2006). There is growing direct evidence that the traditional vitamin D receptor may also have a unique, nontranscriptional role in plasma membrane initiated signalling (Fleet, 2004; Mizwicki

et al., 2004). This would to some extent explain the detection of vitamin D receptors in the cytoplasm of target cells (Feldman et al., 1979; Milde et al., 1989; Barsony et al., 1997; Rougui et al., 1998; Thomas et al., 1999; Prüfer et al., 2000; Walbert et al., 2001; Horst et al., 2003) -- a locus where the receptor protein is synthesized. Classical target organs demonstrating vitamin D receptors are bones, intestines, kidney, liver and skin. The great variety of processes involving calcitriol correlate well with vitamin D receptors detected at several other sites such as: adrenal glands, brain, breast, cardiac muscle, endothelial cells, fibroblasts, lungs, macrophages, pancreas, parathyroid gland, pituitary, skeletal muscle, smooth muscle cells, thyroid glands, T-lymphocytes, urethra and uterus of many species (Berger et al., 1988; Sandgren et al., 1991; Rougui et al., 1998; Sheinin et al., 2000; Chatterjee, 2001; Holick, 2003; Lee et al., 2003). The exact roles of calcitriol at all these sites are not always completely understood.

It is well known that calcitriol plays an important role in the intestine, where it promotes Ca^{2+} resorption via vitamin D receptor-mediated genomic mechanisms. Expression of calbindin-D9k (Haussler et al., 1995; Zhu et al., 1998; Walters et al., 1999; Christakos et al., 2003; Lee et al., 2003; Yamagishi et al., 2006), of its own receptor (Horst et al., 1990; Haussler et al., 1995; Zhu et al., 1998; Chen et al., 2006; Yamagishi et al., 2006) and of Ca-transporting ATPase (CaATPase) (Haussler et al., 1995; Zhu et al., 1998; Yamagishi et al., 2006) are involved.

Calcium metabolism has clinical relevance in high milk-yielding domestic ruminants around parturition, i. e., hypocalcaemia or milk fever may under specific circumstances develop as clinical signs of a disturbed calcium homeostasis (Goff et al., 1995; Horst et al., 2005; Schröder and Breves, 2007). Studies on vitamin D receptor expression in several species generally revealed that vitamin D receptor amounts within the gastrointestinal tract are highest in duodenum and decrease

towards the large intestine (Feldman et al., 1979; Lointier et al., 1991; Kallay et al., 2005). Vitamin D receptors are supposed to be reduced with age (Horst et al., 1990; Duque et al., 2002) and decline during a decrease in blood estrogen concentrations (Liel et al., 1999; Schwartz et al., 2000; Duque et al., 2002). Vitamin D receptors are also more abundant deep in crypts than in villar cells (Clemens et al., 1988). Results are, however, controversial in some instances (Berger et al., 1988; Sandgren et al., 1991; Kinyamu et al., 1997; Wood et al., 1998; Colin et al., 1999; Sheinin et al., 2000; Leonard et al., 2001; Lee et al., 2003; Pazianas et al., 2003; Chen et al., 2006). Until now, no systematic report exists about the distribution pattern of the intestinal vitamin D receptors -- neither in ruminants nor in any other species. Since growth, gestation and lactation are periods of high calcium demand, which may lead to hypocalcaemia, sheep were used in this immunohistochemical study to assess the amount and distribution patterns of vitamin D receptors in small and large intestines of lambs and non-lactating, non-pregnant dams. The results obtained should serve as a basis for currently performed and future experiments in several domestic ruminants during pregnancy and lactation (see Liesegang et al., 2006; Riner, 2006; Singer, 2006).

MATERIALS AND METHODS

Animals

Five lambs (6 months old) and 5 adult, non-lactating and non-pregnant female sheep (1, 2.75, 3, 5 and 5 years of age) were slaughtered at the abattoir of the Vetsuisse-Faculty of the University of Zurich. All lambs were clinically healthy and supernumerary members of the herd of the Institute for Animal Nutrition. Dams were obtained from the Musculoskeletal Research Unit (n=3) or the Clinic for Livestock Animals (n=2) of the Vetsuisse-Faculty of the University of Zurich). Dams were

clinically free of intestinal or skeletal diseases and were killed for experimental reasons during clinical studies.

Tissue Sampling and Processing

Within 30 min after killing with a captive bolt pistol and following bleeding and evisceration, 5 cm long tubular pieces each of duodenum descendens, duodenum ascendens, jejunum, ileum, cecum and colon descendens were collected and fixed in neutral buffered 4% formaldehyde solution for 26 h. Duodenum descendens and duodenum ascendens pieces were taken half way between flexura cranialis and flexura caudalis, flexura caudalis and flexura duodeno-jejunalis, respectively. The jejunal probe was taken from the central part of this segment and the ileal piece cut off half way between the ileal end of the plica ileocecalis and the opening of the ileum into the cecum. A central piece of cecum was harvested and finally a segment of the colon descendens being 30 cm (lambs) or 40 cm (dams) cranial of the anus was isolated from fat tissue and immersed into the fixative. If necessary, intestinal tubules were gently moved in the fixative to remove attached contents and the fixative was renewed if it was soiled with feces. Except for the cecum, which was cut into 3x1 cm pieces after fixation, all tubules were cut into pieces of 1 cm of length and rinsed in tap water for 24 h. This procedure was followed by dehydration in graded ethanol (70, 96 and 100 %), methyl benzoate, xylene and paraffin (60 °C) for 2x4 h each and finally embedding in paraffin (histowax®, Leica Microsystems, Glattbrugg, Switzerland). Sections were cut at 5 µm, mounted on to Superfrost® plus adhesive slides (Menzel-Gläser, Braunschweig, Germany) and dried at 60 °C for 30 min. Haematoxylin and eosin-stained (Böck, 1989) sections were performed to verify physiological state of the tissues and exclude animals possibly exhibiting pathological

changes of the intestine. All tissue specimens of a single animal were run in one batch to minimize intra-animal variation.

Immunohistochemistry

During immunohistochemical procedures, all sections of a single animal were run in a single batch to minimize intra-animal variation. Inter-assay variations thus only may account for differences obtained between animals or groups of animals.

Sections were dewaxed in xylene and isopropyl alcohol, hydrated through serial dilutions of ethanol to water and rinsed in trizma-buffered saline for 2 min (buffer stock solution: 6.1 g trizma base, 50 ml H_2O and 37 ml 1 N HCl, diluted with H_2O to 1000 ml solution, adjust pH to 7.6; working solution: 100 ml buffer stock solution plus 900 ml saline, 0.85 %). Antigen retrieval (Shi et al., 2001) was carried out by incubating the sections in citrate buffer (0.01 mol l^{-1}, pH 6.0; ProTaqs® citrate buffer concentrate and diluted with H_2O to finally obtain 1000 ml solution; Medite, Nunningen, Switzerland) in a microwave oven (600 W, 3 X 5 min). Sections were cooled down to room temperature in citrate buffer for 20 min and endogenous peroxidases inhibited using 3% peroxide for 10 min followed by incubation in TBS for 5 min. All subsequent steps were done at room temperature. Following the avidin-biotin block (20 min each, Vector Laboratories Inc., Burlingame, CA, USA) a serum-free protein block (10 min, DakoCytomation, Zug, Switzerland) was performed in a humidifying chamber. Sections were rinsed in trizma-buffered saline and incubated for 80 min with a biotinylated rat monoclonal antibody (9A7γ, NeoMarkers, Fremont, CA, USA) directed against chicken vitamin D receptor protein (aa 89-105) demonstrating cross-reactivities with sheep and pig antigen (Milde et al., 1989; Schröder et al., 2001). This step was followed by rinsing in trizma-buffered saline (5 min), incubation with StreptABComplex/HRP (30 min, DakoCytomation, Zug,

Switzerland), rinsing in trizma-buffered saline (5 min) and incubation with diaminobenzidine tetrahydrochloride chromogen and H_2O_2 (diaminobenzidine tetrahydrochloride chromogen, Dakocytomation, Zug, Switzerland) for 3 min and rinsing with trizma-buffered saline (5 min). Finally, nuclei were counterstained with hemalaun according to Mayer (Böck, 1989) for 3 min, slides rinsed with tap water for 10 min, dehydrated, cleared in xylene and automatically mounted (Medite, RCM 2000®, Nunningen, Switzerland) in Pertex® (Medite, Nunningen, Switzerland). Negative controls were performed using trizma-buffered saline instead of primary antibodies and positive controls employing duodenal cross-sections of pig (cross-reactivity proven and giving identical results (Milde et al., 1989).

Semiquantitative Evaluation of Histochemical Reactions for vitamin D receptor: Nuclear immunoreactivities were evaluated by a single observer with a binocular microscope (DMLB®, Leica Microsystems, Glattbrugg, Switzerland) equipped with a digital camera for photo documentation (DC 480, Leica Microsystems, Glattbrugg, Switzerland) at a magnification of 400x in several randomly selected microscopic fields. In every tissue specimen nuclear staining intensities of 500 epithelial cells were recorded from following cell types: I. basal crypt epithelial cells; II. intermediate crypt epithelial cells; III. superficial crypt epithelial cells; and IV. surface epithelial cells. Goblet cells were excluded from the counting procedure because they always were devoid of any immunoreaction.

The staining intensities of the nuclei were scored as negative = 0, very weak = 0.5, weak = 1, intermediate = 2, or strong = 3, correlating to absence of brown (that is, blue counterstain only), light brown, brown or dark brown staining, respectively (i.e., staining intensity 0 to staining intensity 3). The frequencies of the different staining intensities were assessed separately for each type of cell -- i.e., basal, intermediate

and superficial crypt epithelial cells and surface epithelial cells -- and were evaluated in each selected microscopic field and then expressed as the sum of positively stained cells of the respective type of cell. Immunoreactivities of the different types of cells in the different locations described above were expressed using a score (vitamin D receptor immunoreactive score, VDR-IRS) that was calculated according to the following equation (Schäubli et al., 2007):

VDR-IRS = 0 x *n*(SI0) + 0.25 x *n*(SI0.5) + 1 x *n*(SI1) + 4 x *n*(SI2) + 9 x *n*(SI3),

where *n* = number of cells counted of a specific type that demonstrated characteristic staining intensities; total number was 500, SI0 = staining intensity negative, SI0.5 = staining intensity very weak, SI1 = staining intensity weak, SI2 = staining intensity intermediate, SI3=staining intensity strong. The calculated immunoreactive score fluctuates between 0 (no specific stain at all) and 4500 (all 500 cells exhibited strong immunoreactivities).

Statistical Analysis

All vitamin D receptor immunoreactive score data for each type of cell are reported as mean ± SEM. Influence of age was calculated in a first step using ANOVA procedure. Differences between cell types were analyzed by a two-factorial analysis of variance for repeated measures (MANOVA, test within subjects) calculated by use of a statistical software program (StatView®, SAS Institute, Cary, NC, USA). The test within subjects indicates whether significant changes occur between different parts of the intestine and the factor cell type tests whether there are differences in vitamin D receptor immunoreactive scores as to cell type, i.e., location of the cells within the mucosa (basal, intermediate or superficial crypt epithelial cells and surface epithelial

cells). Differences were considered statistically significant if the *P*-value was ≤ 0.05. Since several tests were performed for one parameter, a Bonferroni adjustment of the significance level (*P* divided by the number of tests) was performed.

In a further step, Pearson's correlation coefficient between vitamin D receptor immunoreactive scores of duodenum descendens and colon descendens was calculated since amount of vitamin D receptors in colon descendens is used in several studies to estimate amount of vitamin D receptors in the duodenum descendens, the location with highest receptor levels and presumably highest intestinal Ca resorption (Kimberg et al., 1961; Schachter et al., 1961; Schröder et al., 1997; Breves and Schröder, 2005). Data of cell types representing strong and segment-specific vitamin D receptor immunoreactivities, i.e., integrated values of basal and intermediate crypt epithelial cells, were employed. Systat® software (Systat 8.0, Systat Software Inc., Point Richmond, CA, USA) was used for this purpose.

RESULTS

Vitamin D receptor immunoreactivities were evident as brown staining in all segments of the sheep intestines, i.e., duodenum descendens, duodenum ascendens, jejunum, ileum, cecum, and colon descendens (see Fig. 1 A-E). Control sections -- omitting the primary antibody -- were always devoid of any staining (Fig. 2 A-E). Goblet cells -- identifiable by their light supranuclear cytoplasm and their blue counterstained nuclei -- too, were devoid of any specific brown immunostaining (see, e.g., Fig. 1 A_2, D_{2-3}, E_2). This brown coloration was of varying degree and thus indicative of more or less great vitamin D receptor amounts within all other cell types present within the epithelial cell types of crypts (basal, intermediate and superficial crypt epithelial cells) or intestinal luminal surface epithelial cells (see Fig. 1). Nuclear immunostaining was present in all segments of the intestine while cytoplasmic

immunoreactivities were highest in the duodenum (Fig. 1 A_{1-3}) as compared to the more distal segments of the intestine, which could exhibit faint or even no cytoplasmic immunoreactivities (Fig. 1 B-E). Cytoplasmic immunoreactivities were also higher in the depth of crypts and decreased towards the openings of the crypts and the surface epithelium (Fig. 1 A, B). A generally parallel decrease in nuclear immunoreactivities (Fig. 1 A, B), which were, however, in some segments less clearly expressed (Fig. 1. C-E), could also be recognized by the use of the light microscope. These differences were also reflected by corresponding nuclear vitamin D receptor immunoreactive scores. Vitamin D receptor immunoreactive score data of lambs did not differ significantly from data obtained from dams (F-value = 0.035, $p = 0.86$), thus all data were compiled and evaluated statistically as a whole. As outlined in table 1, vitamin D receptor immunoreactive sores of basal crypt epithelial cells decreased significantly from high levels in duodenum to lower ones in more distal segments of the intestine (duodenum descendens and ascendens > jejunum, ileum, cecum and colon descendens; jejunum > cecum; $p<0.05$). This was also true for intermediate crypt epithelial cells (duodenum descendens > jejunum, ileum, cecum and colon descendens; duodenum ascendens > ileum and cecum; $p<0.05$). Superficial crypt epithelial cells and surface epithelial cells did not exhibit statistically significant differences in immunohistochemical coloration for vitamin D receptors between intestinal segments ($p>0.05$).

Differences between the several cell types -- basal, intermediate and superficial crypt epithelial cells and surface epithelial cells -- within an intestinal segment were significant for duodenum descendens (basal and intermediate crypt epithelial cells > superficial crypt epithelial cells and surface epithelial cells; $p<0.05$), duodenum ascendens (basal crypt epithelial cells > intermediate and superficial crypt epithelial cells and surface epithelial cells; intermediate crypt epithelial cells > superficial crypt

epithelial cells and surface epithelial cells; $p<0.05$), jejunum (basal crypt epithelial cells > superficial crypt epithelial cells and surface epithelial cells; intermediate crypt epithelial cells > surface epithelial cells; $p<0.05$) and colon descendens (intermediate crypt epithelial cells > surface epithelial cells; $p<0.05$), while in ileum and cecum no such discrepancies could be detected ($p>0.05$).

Vitamin D receptor immunoreactive scores of duodenum descendens and colon descendens (i.e., basal and intermediate crypt epithelial cells) were significantly correlated ($r = 0.94$; $p<0.05$).

Discussion

The results of the present immunohistochemical study in sheep clearly indicate for the first time, that vitamin D receptor protein exhibits a distinct nuclear distribution pattern in every segment of sheep intestines. There was a significant gradient in nuclear vitamin D receptor immunoreactive scores of basal and intermediate crypt epithelial cells from maximum values in duodenum and jejunum to minimal values in ileum, cecum and colon. Furthermore location-specific patterns within the mucosa were visible within each segment of the intestine. Strongest nuclear (and also cytoplasmic) immunoreactions were recorded in basal and intermediate crypt epithelial cells and decreased in superficial crypt epithelial cells and along the surface epithelium. This gradient was, however, only clearly visible in the duodenum and jejunum, was absent in the ileum and cecum and was present in a slightly modified version in the colon descendens. Thus it correlated well with stronger nuclear (and cytoplasmic) immunoreactions found in the more proximal parts of the small intestine, i.e., duodenum and jejunum versus ileum, cecum and colon. Surface epithelial cells and superficial crypt epithelial cells did not exhibit any significant differences in nuclear vitamin D receptor immunoreactive score (and cytoplasmic

immunoreaction) and therefore represented an intestinal epithelial surface uniformly provided with little vitamin D receptor amounts. The distribution pattern of vitamin D receptor protein demonstrated in this study was in good general accordance with several reports on I. vitamin D receptor protein or vitamin D receptor mRNA expression (Feldman et al., 1979; Clemens et al., 1988; Lointier et al., 1991; Yamamoto et al., 1999; Kallay et al., 2005), II. calbindin expression (Barley et al., 1999; Yamagishi et al., 2002), III. epithelial calcium transporter (Barley et al., 2001), and IV. segment-specific calcium resorption or influx (Kimberg et al., 1961; Schachter et al., 1961; Schröder et al., 1997; Breves and Schröder, 2005). It should, however, also be considered that other factors such as nuclear co-activators or co-repressors additionally may influence calbindin expression or calcium absorption rates (Fretz et al., 2006; Kim et al., 2006; Zella et al., 2006). In these studies cited here each parameter was evaluated based on one or two intestinal segments only or was done with just one or two species. In contrast, there are a few human studies that observed no differences in the amount of vitamin D receptor between the various segments of the small intestine (Berger et al., 1988; Pazianas et al., 2003) and rats (Chen et al., 2006). Thus, the results presented here close a great gap in the knowledge of vitamin D receptor distribution within the intestine in general.

In a recent semiquantitative study Wilkens (2006) confirmed the expression of Ca resorption-associated proteins such as Ca^{2+} channel proteins TRPV 5 and 6, calbindin D_{9k}, Ca^{2+}-ATPase (PMCA) and Na^{+}/Ca^{2+} exchanger (NCX) in duodenum and jejunum of the sheep using RT-PCR, in situ hybridization and immunohistochemistry. Tests for calbindin D_{28K} were negative.

It is interesting to note that most intense vitamin D receptor immunoreactions were confined to lower parts of the crypts in the duodenum and jejunum. This is in

accordance with the segments of maximum intestinal calcium uptake in sheep (Kimberg et al., 1961; Schachter et al., 1961; Schröder et al., 1997; Breves and Schröder, 2005). It may therefore be speculated that differentiation of post-mitotic cells to cells exhibiting above-average calcium absorbing capacities is correlated with above-average vitamin D receptor expression rates. Via vitamin D receptor mediated genomic mechanisms calcitriol should provide a calcium absorbing machinery of maximum capacity, i.e., high contents of calbindin-D9k (Haussler et al., 1995; Zhu et al., 1998; Walters et al., 1999; Christakos et al., 2003; Lee et al., 2003; Yamagishi et al., 2006), of its own receptor (Horst et al., 1990; Haussler et al., 1995; Chen et al., 2006; Yamagishi et al., 2006) and of Ca-transporting ATPase (Haussler et al., 1995; Zhu et al., 1998; Yamagishi et al., 2006) as already confirmed for some of these parameters in several studies (Feldman et al., 1979; Clemens et al., 1988; Lointier et al., 1991; Barley et al., 1999; Yamamoto et al., 1999; Barley et al., 2001; Yamagishi et al., 2002). Finally, the quantitative role of intestinal Ca absorption in domestic ruminants is discussed controversially because it was shown that the rumen is an important site for active and calbindin-9kD-independent Ca absorption in sheep (Schröder et al., 1997, 1999, 2001; Wilkens, 2006; Leonhard-Marek et al., 2007; Schröder and Breves, 2007).

Stem cells are supposed to be located near the base of the crypts of the small intestine and daughter cells migrate into two directions: prospective Paneth cells and endocrine cells differentiate during a downward movement to the base of crypts and reside there for approximately 20 days. Prospective enterocytes and goblet cells migrate -- while initially dividing several times and thus forming a transit cell population -- in approximately 3 - 5 days to the surface (Attaix and Meslin, 1991; Ferraris et al., 1992; Potten and Grant, 1998; Gassler et al., 2002; Marshman et al.,

2002; Boshuizen et al., 2003; Pinto and Clevers, 2005). The process of cellular differentiation is supposed to start immediately after mitotic generation of the cells. Since highest vitamin D receptor immunoreactive scores were found in basal and intermediate crypt epithelial cells, calcitriol should not only be involved in differentiation and maturation of daughter cells but may also be involved in the regulation of cellular homeostasis at this site. The function of calcitriol in Paneth cells and endocrine cells remains to be elucidated. It is interesting to note that superficial crypt epithelial cells and surface epithelial cells exhibited a uniformly basal vitamin D receptor immunoreactive score along the whole intestine. Thus, when enterocytes and goblet cells reach the surface of the intestine and absorb calcium, they are no longer under strict control of calcitriol. That means, vitamin D-regulated calcium absorption should be regulated in the depth of the crypts where maturing cells are prepared for specific absorption of calcium, which takes place later, when cells have reached the intestinal surface. Thus, there is only a very narrow window of time and localization, where calcitriol can regulate cellular function in the intestine.

In the large intestine, stem cells are located at the base of the crypts and all daughter cells migrate upwards during differentiation (Potten and Grant, 1998; Backus et al., 2002; Marshman et al., 2002; Pinto and Clevers, 2005). This implies that preferably cellular differentiation is regulated by calcitriol, because highest vitamin D receptor immunoreactive scores are recorded mainly in intermediate (cecum and colon descendens) or also superficial crypt epithelial cells (cecum only).

In the present study, no age-dependent differences in vitamin D receptor immunoreactive scores could be detected. It should be considered, however, that no senile dams were included in the present study. Furthermore, since specimens of single animals were run in a single batch, inter-assay variations may partially account

the lack of age-dependent differences. For this reason no conclusions about pathogenesis of osteoporosis in humans may be drawn. This lack of age-dependent differences is in accordance with findings -- gained with identical study design and methodology -- in cattle and goat (Riner, 2006; Singer, 2006) and several other reports (Kinyamu et al., 1997; Wood et al., 1998; Lee et al., 2003; Pazianas et al., 2003; Yamagishi et al., 2006) and in contrast to data published elsewhere (Horst et al., 1990; Ebeling et al., 1992; Liang et al., 1994; Bischoff et al., 2001; Duque et al., 2002; Holick, 2003). Since an age and/or estrogen dependent impairment of calcium resorption was detectable in elder subjects in numerous studies, a resistance to vitamin D was postulated (Kinyamu et al., 1997; Wood et al., 1998) and furthermore vitamin D-dependent (Kinyamu et al., 1997; Liel et al., 1999; Schwartz et al., 2000; Leonard et al., 2001; Duque et al., 2002; Gilad et al., 2005) and independent effects of estrogens on calcium resorption were verified (Colin et al., 1999; Eisman, 2001; Van Cromphaut et al., 2003). Thus, in future studies hormone- and age-associated effects on calcium and vitamin D metabolism in sheep should be re-examined also in senescent dams with and without hormone substitution and evaluated separately. To minimize inter-assay variations in such experiments, slides of young and old animals should be processed in a single batch and be assessed comparatively.

In studies on ruminant calcium homeostasis, biopsies of colon descendens have been taken and assessed for vitamin D receptors (Goff et al., 1995). Parturition is paralleled by a drop on vitamin D receptors in the colon, which is supposed to be representative for the amounts of vitamin D receptors in the duodenum (Goff et al., 1988). The significant positive correlation detected between the vitamin D receptors immunoreactive scores of the duodenum and colon in the present study support this view. Results of our group (Liesegang et al., 2006), which were obtained in sheep

and goat during late gestation and during lactation, confirm Goff's observations made in 1995.

The results of the present study indicate that vitamin D receptors are expressed in segment- and localization-specific patterns within sheep intestines. Vitamin D receptor immunoreactive score correlates well with assumed differing capacities of active intestinal calcium resorption. Vitamin D receptor is expressed at sites of cell proliferation and differentiation; thus it is supposed also to be involved in the regulation of these processes. Further studies -- including other species -- are needed to elucidate the specific role of the vitamin D receptors in gastrointestinal calcium absorption under different circumstances and to get insights into species-specific mechanisms involved.

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TABLE 1. Vitamin D receptor immunoreactive scores (mean ± SEM) of the various cell types within the epithelial layers of the different segments of sheep intestine

	crypt epithelial cells			surface epithelial cells
	basal	intermediate	superficial	
duodenum descendens	$1362 \pm 148^{a,1}$	$1015 \pm 123^{a,1}$	405 ± 107^{2}	273 ± 75^{2}
duodenum ascendens	$1271 \pm 109^{a,1}$	$817 \pm 110^{a,b,2}$	351 ± 102^{3}	262 ± 72^{3}
jejunum	$724 \pm 101^{b,1}$	$495 \pm 76^{b,c,1,2}$	$257 \pm 72^{2,3}$	183 ± 48^{3}
ileum	$426 \pm 148^{b,c,d}$	$267 \pm 60^{c,}$	236 ± 62	192 ± 59
ceceum	$178 \pm 63^{c,d}$	$344 \pm 75^{c,}$	377 ± 106	183 ± 65
colon descendens	$275 \pm 47^{b,c,d,1,2}$	$482 \pm 85^{b,c,1}$	$264 \pm 62^{1,2}$	174 ± 41^{2}

Mean values with different superscripts (letters) within a column -- i.e., between the different intestinal segments differ significantly ($p<0.05$).

Mean values with different superscripts (cyphers) within a line -- i.e., between the different epithelial cell types differ significantly ($p<0.05$).

FIGURE LEGENDS

Fig. 1. Vitamin D receptor immunohistochemistry in sheep duodenum descendens (A_{1-3}), jejunum (B_{1-3}), ileum (C_{1-3}), cecum (D_{1-3}) and colon descendens (E_{1-2}); Vitamin D receptor immunoreaction is demonstrated as brown staining and contrasts well with blue counterstain of nuclei. Duodenum exhibits a clear basal to superficial gradient within the mucosa (A_{1-3}). Basal and intermediate crypt epithelial cells (BC, IC) demonstrate strong nuclear and cytoplasmic immunoreaction (A_1, A_3), while superficial crypt epithelial cells (SC) and surface epithelial cells (S) exhibit only very weak immunostaining (A_{1-2}). Goblet cells (GC, labelled only in A_2, D_{2-3}, and E_2) are always devoid of vitamin D receptor protein. Jejunum exhibits a less distinct basal to superficial gradient (B_{1-3}), because BC demonstrate only an intermediate immunoreaction (B_1, B_3). In ileum no such distinct gradient is evident (C_{1-3}), i.e., all crypt and surface epithelial cells - except immuno-negative goblet cells - demonstrate a weak immunoreaction (C_{1-3}). Large intestines (D_{1-3}, E_{1-2}) generally exhibit weak immunoreactions. Intermediate crypt epithelial cells (IC) exhibit stronger immunoreactions compared to BC, SC and S in cecum (D_{1-3}) and colon descendens (E_{1-2}). Scale bar (left upper corner of A_1) represents 100 µm in A_1, B_1, C_1, D_1 and E_1, 50 µm in A_{2-3}, B_{2-3}, C_{2-3} D_2, and 45 µm in D_3 and E_2. VS, stroma of intestinal villi.

Fig. 2. Controls omitting the primary antibody; sections shown are from the same tissue blocks as demonstrated in Fig. 1. Upper panel (A_1-E_1, scale bar represents 100 µm) exhibits an overview over the whole mucosa and the lower panel (A_2-E_2, scale bar represents 10 µm) visualises basal crypts at a higher magnification to document the complete absence of any immunoreaction. $A_{1,2}$, duodenum descendens; $B_{1,2}$, jejunum; $C_{1,2}$, ileum; $D_{1,2}$, cecum; $E_{1,2}$, colon descendens.