Lactase, sucrase-isomaltase, and carbamoyl phosphate synthase I expression in human intestine

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Chapter 6

Regulation of Lactase and Sucrase-Isomaltase Gene Expression in the Duodenum during Childhood

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Chapter 6

Regulation of Tricere and Solute-Transporting Gene Expression in the Developing Nephron: Children

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Regulation of Lactase and Sucrase-Isomaltase Gene Expression in the Duodenum during Childhood

Abstract

Background: In children, lactase and sucrase-isomaltase are essential intestinal glycohydrolases, and insufficiency of either enzyme causes diarrhea and malnutrition. Little is known regarding the regulation of lactase and sucrase-isomaltase expression in the duodenum during childhood. Therefore, we studied the mechanisms of regulation of duodenal expression of both enzymes in a study population with ages ranging from 1 to 18 years. Methods: Duodenal biopsies from 60 Caucasian children were used to analyze tissue morphology, and to quantify lactase and sucrase-isomaltase mRNA and protein. Results: Among healthy individuals, we found high inter-individual variability in both mRNA and protein levels for lactase and sucrase-isomaltase. Lactase mRNA level per individual did not correlate with sucrase-isomaltase mRNA level and thus appeared independent. Both lactase and sucrase-isomaltase protein levels correlated significantly with their respective mRNA levels. For each enzyme, we found a significant inverse correlation between the degree of villus atrophy and mRNA levels. Aging from 1 to 18 years old did not result in significant changes in mRNA or protein levels of either enzyme. Immunostaining patterns within the duodenal epithelium for lactase differed from sucrase-isomaltase in adjacent sections illustrating independent regulation at the cellular level. Conclusions: In the duodenum of Caucasian children, lactase and sucrase-isomaltase seem primarily regulated at the transcriptional level. The expression of each enzyme in the intestinal epithelium is regulated by an independent mechanism. Lactase and sucrase-isomaltase exhibit stable mRNA and protein levels in healthy children during ageing to adulthood. Mucosal damage affected levels for both enzymes negatively.

Introduction

Although gene expression of lactase (EC 3.2.1.23/45/46/62) and sucrase-isomaltase (EC 3.2.1.10/48) has been studied extensively in human adults, human cell lines, isolated cells, rodents, rabbits and pigs (Reviewed in Van Beers et al, 1995), little is known about their regulation in humans during infancy and childhood. Lactase and SI are anchored in the brush border membranes of the intestinal enterocytes and are crucial in the digestion of dietary carbohydrates (Van Beers et al, 1995). Lactase is the sole enzyme in the intestine that is capable of hydrolysing lactose from milk, which is the primary energy source for newborn children. Sucrase-isomaltase is essential in the final hydrolysis of starch, and becomes essential somewhat later in human life, when starch has become the predominant carbohydrate source. Low levels of either enzyme are often associated with diarrhea and malnutrition, and may affect growth and development of children. Two forms of deficiencies are defined for these enzymes: primary deficiency, that is governed by specific temporal regulation of the
enzyme gene, and secondary deficiency that is caused by mucosal damage, leading to loss of functional enterocytes that express these enzymes. In very rare cases, primary enzyme deficiency may result from mutations in the coding region of the gene, leading the expression of non-functional proteins.

Endoscopic mucosal sampling is a valuable and established diagnostic procedure in the diagnosis of upper gastrointestinal complaints, including measurement of lactase and SI enzyme levels (Black et al., 1988; Dandalides et al., 1989; Newcomer and McGill, 1966, 1967; Savilahti et al., 1983; Lojda et al., 1984; Olsen et al., 1996; Welsh et al., 1978). Duodenal biopsies are well suited to study the expression of these enzymes, since it was established that there are only minor quantitative differences in lactase and SI enzyme levels in duodenum, compared to the jejunum (Dandalides et al., 1989; Bergoz et al., 1981). Both enzymes are essential for the growth and development of children, and although lactase and SI have been studied in conjunction in human cell lines and in rat intestine (Van Beers et al., 1995; Rings et al., 1994), there are very few reports that simultaneously studied the regulation of lactase as well as SI in the human duodenum during post-natal development. Therefore, in this study, we have measured lactase and SI mRNA as well as protein levels in endoscopically obtained biopsies from the distal half of the duodenum of Caucasian children between 1-18 years old. From these data we will describe the mechanisms of regulation of lactase and SI expression from infancy to adulthood.

Materials and methods

Patients, tissue, and ethical considerations

In a prospective study, duodenal forcipal biopsies were taken from 60 Dutch Caucasian children, who attended the pediatric gastroenterology unit because of various upper gastrointestinal complaints. Each patient underwent gastro-duodenal endoscopy and duodenal biopsy for diagnostic purposes. We obtained permission to take two extra biopsies per patient from the distal region of the duodenum, which was ethically considered the maximum for this study. Biopsies were taken with informed consent of the patients and their parents, and with permission of the medical ethical committee of our institution. All children were of ethnic Dutch origin, a homogeneous population that is known to maintain high levels of lactase throughout lifetime (Flatz, 1987).

Biopsies were taken distally to the papilla of Vater and proximally of the ligament of Treitz. One biopsy was diagnosed by a pathologist as independent evaluation of tissue morphology, as part of the normal diagnostic workup. A second biopsy was used by us to isolate RNA, and another was paraformaldehyde-fixed for (immuno-) histochemistry. For each patient, age, clinical symptoms, and medical history were recorded. In 40 patients ranging from 1 to 18 years, duodenal biopsies could be analyzed for the following parameters; mucosal morphology, lactase and SI mRNA levels, and lactase and SI protein levels. Biopsies of the remaining 20 patients
were subjected to immunohistochemistry and morphology measurements, but not used for mRNA quantification.

**Antibodies**

We used an anti-human lactase monoclonal antibody (Mab) HBB 1/90/34/74 and two anti-human SI Mabs, HBB 2/219/20 and HBB 2/219/88. The latter anti-SI Mabs gave identical results in each patient studied. Each of the above Mabs was described to recognize the precursor proteins as well as mature, brush border proteins (Hauri et al., 1985). As a negative control, ascites was used prepared in sibling mice containing a Mab against rat lactase (Quaroni and Isselbacher, 1985), which does not cross-react with human lactase. Three independent anti-human lactase Mabs prepared from hybridoma supernatant (mLac1, mLac4, and mLac5) were also used as controls in some experiments (Maiuri et al., 1991). Intestinal alkaline phosphatase was detected in biopsy sections by an anti-human intestinal alkaline phosphatase Mab (IgGl), that was affinity purified and supplied in PBS buffer (clone MIG-I15, Monosan, Sanbio).

**Morphology**

The morphology of the duodenal mucosa, also referred to as crypt/villus ratio, was assessed double blind using light microscopy at low magnification (100x), giving scores ranging from 0 for total villus atrophy, 1 for moderate villus atrophy (ratio villus/crypt < 1), 2 for mild villus atrophy, to 3 for normal morphology according to established criteria (Mercer et al., 1990). Our findings were checked against the analysis of the pathologist of a duplicate biopsy of each patient.

**Immunohistochemistry**

Seven micrometer thick sections from 4% paraformaldehyde-fixed, paraffin-embedded biopsies were deparaffinated, incubated for 30 min in 3% H2O2 in PBS to inactivate endogenous peroxidase activity, 30 min in a solution of 10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, (pH 7.4) to prevent background staining. The sections were incubated overnight with adequate dilutions in PBS of the primary antibodies, 1.5 h with rabbit-anti-mouse serum (1:7500, DAKO, Glostrup, Denmark), 1.5 h with goat-anti-rabbit serum (1:1000, DAKO) and with rabbit peroxidase-anti-peroxidase (1:1000, DAKO). All incubations were at room temperature. Anti-lactase (HBB 1/90/34/74) and anti-SI (HBB 2/219/20 and HBB 2/219/88) Mabs were diluted 1:1000 from ascites (Hauri et al., 1985). Presence of immunohistochemical staining was determined using light microscopy at 400x magnification. Furthermore, we distinguished four semi-quantitative classes of staining intensity, that were scored on the staining of the villus brush border; 0, absence of staining; 1, very weak staining; 2, evenly distributed moderate staining; and 3, evenly distributed dark staining. 'Patchy' staining was observed in immunostained tissue sections of some patients. In these cases a small number of villus enterocytes showed a clear positive immunostaining for either lactase or SI, that was seemingly random distributed, amidst large numbers of negative cells. Since only few cells were positive in these sections, this type of immunostaining was semi-quantitatively scored.
as type 1 staining. Semi-quantitative measurements of staining intensities were only assessed in villus brush border of biopsy sections that had been immunostained in triplicate in separate experiments. All sections were examined at least twice by two investigators, in a double-blinded fashion.

**mRNA quantification**

RNA was isolated from individual biopsies using the RNeasy protocol (Qiagen, Chatsworth, CA). mRNA quantification was performed on spot blots using specific DNA probes synthesized using random hexamer primers, incorporating $\alpha^{32}P$-dATP to at least $1 \times 10^8$ cpm/µg (Sambrook *et al.*, 1989). Lactase probes were synthesized from the 6.3 kb full length human lactase cDNA (Nairn *et al.*, 1991), SI probes from 428 bp of a human SI clone (Van Beers *et al.*, 1995), and glyceraldehyde phosphate dehydrogenase (Gapdh) from a 1 kb fragment of human Gapdh cDNA (Tokunaga *et al.*, 1987). Between 100-500 ng of intact RNA as determined by agarose gel electrophoresis and ethidium bromide staining (Not shown) from each biopsy was blotted onto positively charged Qiabrine filters (Qiagen), dried and baked at 80°C for 2 h. Prehybridization was performed at 65°C for 1 h in SSC (i.e. 7% SDS, 0.1 mM EDTA, 0.5 M Na$_2$HPO$_4$, pH 7.2). Hybridization was performed for 17 h at 65°C. Filters were washed at 65°C twice 20 min in 6xSSC, 0.1% SDS, once 20 min in 3xSSC, 0.1% SDS and once 20 min in 0.1xSSC, 0.1% SDS. Autoradiographs were prepared using Phosphorimage screens (Molecular Imaging), that were exposed to hybridized membranes for 48 h, and quantified (in a.u.) using a PhosphorImager and ImageQuant software (Molecular Imaging). Membranes were stripped of adherent probe by washing at 80°C in 0.1% SDS for several h before hybridization with each next probe. Stripped blots were checked for absence of radioactivity by exposure to PhosphorImager screens for 48 h. Sequentially, all the blotted RNA samples have been incubated with probes for SI mRNA, lactase mRNA and Gapdh mRNA. For each individual RNA sample, signals detected for lactase and SI mRNAs were normalized to Gapdh mRNA and therefore corrected for sample size.

**Reproducibility**

Results in this study were from two biopsies from each patient, one for mRNA isolation and one for immunohistological measurements. However, we have previously shown the reproducibility of our methods, since very similar results were obtained in duplicate biopsies from the duodenum, even when biopsies were compared from different regions of the duodenum (Van Beers *et al.*, 1998).

**Statistics**

Quantitative data were analyzed by statistical analysis using SAS 6.11 software. Upon the presumption that the variables were continuous but not distributed normally, a Spearman correlation analysis was performed (compare figure 2 and 3). When, in addition to a non-normal distribution, one of the variables was non-continuous a non-parametric Kendall Rank correlation analysis of significance was performed (compare figure 1 and 4). We defined statistical significance at $p<0.05$. 


Results

Lactase and SI levels are independently expressed

For meaningful comparison between mRNA levels of lactase and SI, our calculations were based on identical tissue base (denominator). Therefore, we performed RNA spot-blots since these enabled multiple sequential probe hybridizations on the same RNA samples. Based on these quantifications we calculated the ratio of lactase and SI mRNA amounts, and plotted this against the age of the individuals (figure 1). The ratios between lactase and SI mRNA among healthy individuals show a wide variation, that appeared statistically unrelated to the age of the individuals (r=-0.003, p=0.99). Therefore, we concluded that levels of lactase mRNA were independent from levels of SI mRNA in the duodenum during childhood.

Figure 1. Lactase and SI mRNA levels are independently regulated. Correlation analysis of the ratio of lactase and SI mRNA levels versus the age of 28 Dutch Caucasian children with normal duodenal mucosa. Lactase and SI mRNAs were each detected with specific probes sequentially hybridized to the same blot. Each point in the graph represents the ratio of the mRNA levels for lactase and SI detected in RNA isolated from one patient. Statistical significance was considered using the Spearman correlation analysis (r=-0.003, p=0.99).

Lactase and SI are primarily transcriptionally regulated

To assess the primary level of gene regulation of lactase and SI, we measured the amounts of lactase and SI protein semi-quantitatively by immunohistochemistry, and the levels of their respective mRNAs (figure 2). The correlations between lactase or SI protein levels with their respective mRNA amounts proved statistically significant (p=0.023 and p=0.006, respectively), indicating primary transcriptional control of expression for both genes. Nonetheless, there remains a large inter-patient variability in the levels of the respective mRNAs, which is reflected in the correlation coefficient; 0.27 for lactase and 0.33 for SI. Despite the inter-individual variation, the statistical analyses convincingly showed that both lactase and SI mRNA amounts have significant predictive value for the levels of their respective proteins in the villus brush border of the duodenum.
Figure 2. Correlation between levels of lactase and SI proteins and their respective mRNAs. Panel A shows the correlation between lactase mRNA (in a.u.) and immunodetectable lactase protein. Panel B shows the correlation between SI mRNA (in a.u.) and immunodetectable SI protein. The assignment of the semi-quantitative protein levels (0, 1, 2 and 3) is explained in the methods section. Each point represents measurements from the duodenal biopsies of one of 40 Caucasian children. Statistical significance was assessed using the Kendall Tau rank correlation test (panel A; r=0.27, p=0.023 and panel B; r=0.33, p=0.006).

**Lactase and SI are adversely affected by mucosal damage**

We analyzed the relation between lactase and SI mRNA levels and the degree of villus atrophy in our child population (figure 3). Within single mucosal morphology classes a wide range of mRNA levels was observed for either enzyme. Nevertheless, statistical analysis for both lactase and SI mRNA showed highly significant inverse correlation with the mucosal damage (r=0.54, p=0.0001 and r=0.41, p=0.0009, respectively).

**Levels of lactase and SI in Caucasians do not change during post-natal development**

We determined the profiles of lactase and SI gene expression during childhood, by analyzing the amounts of the mRNAs for each enzyme in individuals without mucosal abnormalities in the duodenum in relation to the age of the individuals (figure 4). Statistical analysis showed no significant relation between age and lactase mRNA levels (r=-0.18; p=0.37) or between age and SI mRNA levels (r=0.10; p=0.63).
Figure 3. Effects of villus atrophy on lactase and SI mRNA levels. Correlation of lactase mRNA and mucosal morphology (A), and SI mRNA and mucosal morphology (B) in duodenal biopsies from 40 Caucasian children. Morphology was scored as 0-3, (see methods). Significance was calculated by Kendall Tau rank correlation test (A, r=0.54, p=0.0001; B; r=0.41, p=0.0009).

Figure 4. Expression of lactase and SI mRNA during childhood. A shows the correlation analysis between lactase mRNA (in a.u.) and age and B between SI mRNA (in a.u.) and age in biopsies from 28 Caucasian children with normal duodenal mucosal morphology. Statistical significance was calculated by the Spearman correlation analysis (panel A, r=-0.18, p=0.37 and panel B; r=-0.10, p=0.63).
In the same 28 individuals as presented in figure 4 with normal mucosal morphology, as well as in a further 14 individuals with unaffected mucosa, we analyzed the semi-quantitative immunohistochemical detection of the lactase and SI proteins in relation to age. Statistical analysis showed, similar to the mRNA data, no correlation for lactase or SI protein levels with age (lactase; \( r=-0.06, p=0.41 \), and SI; \( r=0.11, p=0.36 \)) (Not shown).

**Expression of lactase and SI at the cellular level**

We investigated the lactase and SI expression at the microscopical level in some more detail. The independent levels of expression of lactase and SI in healthy duodenum appeared to be explained, at least in part, by differential expression of lactase and SI at the cellular level. Immunostaining of biopsy sections revealed large variations in levels as well as patterns of lactase and SI protein expression. Among the patients without any mucosal abnormalities \((n=42)\), four combinations of immunostaining were distinguished: 1. lactase present and SI absent \((2/42)\), 2. lactase absent and SI present \((4/42)\), 3. both proteins absent \((1/42)\), and 4. both proteins present \((35/42)\). Figure 5 illustrates the most prevalent phenotype among individuals with normal mucosa in which both proteins are present \((83\%)\). Lactase was detected from the crypt-villus junction to almost the tips of the villi, whereas SI was found very deep in the crypts and reached up to approximately one-third of the villus length (figure 5A and B). In sections of a biopsy of another child expression was demonstrated of both lactase and SI along the entire length of the villus, while SI was also detected in crypt brush border (figure 5C and D). These examples represent a remarkable difference between immunodetection of lactase and SI in the brush border of crypts. In the majority of children SI was detected in the brush border of crypt enterocytes \((83\%\) of SI-positive patients, e.g. figure 5B and D), while lactase was never detected in the crypts (compare figure 5A and C).

Figures 6A and B illustrate independent expression of lactase and SI among duodenal enterocytes. Within the epithelium, lining a single villus, lactase was sometimes expressed in enterocytes that lack SI expression. Often, abrupt transitions (within one cell position) from positive to negative staining was observed (figure 6B). Also the reverse situation occurred, when SI was present in cells that were negative for lactase (Not shown).

Strikingly, in 16 out of 60 patients \((27\%)\) studied by immunohistochemistry both the anti-lactase and the anti-SI Mabs stained an intracellular supranuclear region, in addition to the commonly observed brush border staining (figure 6C and D). In individuals positive for this intracellular staining, high amounts of intracellular lactase as well as intracellular SI were detected in adjacent sections. No cases were found in which only one, but not the other, Mab stained intracellularly. The intracellular staining was found in all cells expressing lactase and SI on their brush borders, in villus as well as in crypt enterocytes, whereas staining was not detected in goblet and Paneth cells. This phenotype, i.e. concomitant high intracellular lactase and SI levels, did not correlate with morphology of the tissue, since it was also observed in mucosae.
of particular patients with villus atrophy (Not shown). Moreover, there was no correlation with the age, sex, or ABO-blood group of the individual (Not shown).

Figure 5. Immunohistochemical detection of lactase and SI protein in duodenal biopsies. Photos show representative immunodetection on sections of duodenal mucosal biopsies from two children with normal mucosal morphology. In both cases, lactase (panels A and C) or SI (panels B and D) are shown in closely adjacent sections of the same biopsy. Original magnification 100x.

Figure 6. Expression of lactase and SI at the cellular level. Panels A and B illustrate independent expression of lactase and SI among enterocytes. Lactase is expressed in enterocytes that lack SI expression (arrow). Note the abrupt transition from positive to negative staining for SI compared to the continuous staining for lactase (arrowhead). Panels C, D and E illustrate the intracellular staining for lactase and SI noted in 27% of the children. In panel C high amounts of intracellular lactase are detected in all enterocytes along the villus, and panel D shows the same phenomenon in an adjacent section stained for SI. Panel E shows staining of an adjacent section using an anti-intestinal alkaline phosphatase Mab. Original magnification 400x.
We performed a number of control experiments to find an explanation for this phenotype. The intracellular staining was observed indistinguishably for each of the two anti-human SI Mabs, but not for the anti-brush border enzyme alkaline phosphatase Mab (figure 6E). Three independent anti-human lactase Mabs: mLac1, mLac4, and mLac5, did not detect this intracellular lactase (Not shown). Negative controls included ascites prepared in sibling mice containing a Mab against rat lactase (Quaroni and Isselbacher, 1985), which has been shown not to cross-react with human lactase both in immunohistochemistry and immunoprecipitation (Not shown), indicating that we detected indeed intracellular lactase using the anti-human lactase Mab. Hybirdoma culture supernatants of the Mabs (i.e. HBB 1/90/34/74, HBB 2/219/20, and HBB 2/219/88) gave the same results as ascites, and also after purification from ascites, using affinity chromatography on protein A-Sepharose, these Mabs gave the same results (Not shown). The secondary antibodies were not responsible for the intracellular staining, since omission of the first antibodies (i.e. the Mabs) from the immunohistochemical procedures, resulted in perfectly blank control staining (Not shown). We also tested two additional fixation protocols for biopsies (that is, occasionally, and just for this purpose): fixation in methanol/acetone/water (40/40/20, v/v/v), or the use of cryosections. If the intracellular staining phenotype was observed in the standard paraformaldehyde-fixed biopsy sections, then this staining was also observed in sections of the alternatively treated tissue (Not shown).

Discussion

Lactase and SI are both essential glycohydrolases for the development of the growing child, since these enzymes account for almost all disaccharide hydrolysis in humans. Lactase is particularly important in suckling children, whereas SI becomes more important later in life when milk is no longer the sole nutrient. However, there is very little specific knowledge of regulation of expression of the enzymes in the duodenum during human post-natal development. In this study, we quantified lactase and SI expression in conjunction, and we were able to assess the levels of regulation that govern the expression of these important genes during childhood.

Lactase and SI genes are independently regulated

There is substantial evidence from a variety of species, especially rat (Rings et al., 1994; Krasinski et al., 1994), that lactase and SI are mainly regulated at the transcriptional level (Van Beers et al., 1995). Here, we analyzed quantitative data for both these glycohydrolases in the duodenum of a group of children of various ages. Quantitative analysis of gene regulation in the intestine during development is generally hampered by the lack of reference to suitable tissue parameters, since parameters like size, dry or wet weight, cell-type composition, and DNA content change continuously in a growing individual. We have circumvented this partly by analyzing the ratio of lactase mRNA and SI mRNA levels, which had both been derived by identical methods from the same biopsy. The results clearly indicate that
the lactase and SI genes are expressed independently in the duodenum during childhood, suggesting that the mechanisms that regulate their expression are only distantly related. This is in accordance with earlier studies, which indicated that the promoter sequences of the respective genes are quite distinct (Traber et al., 1992; Boll et al., 1991).

**Lactase and SI are primarily transcriptionally regulated during childhood**

We described lactase as well as SI regulation in children based on steady-state levels of mRNA and protein. Other studies had already convincingly shown a linear relationship between the amount of glycohydrolase protein and the corresponding enzyme activity (Harvey et al., 1985; Fajardo et al., 1994; Rossi et al., 1997). Levels of lactase or SI mRNA, protein and in some studies also enzyme activities have been measured in adult humans, and in rat and rabbit (Savilathi et al., 1983; Fajardo et al., 1994; Rossi et al., 1997; Rousset et al., 1989; Nichols et al., 1992; Lloyd et al., 1992; Rubin et al., 1992; Traber et al., 1992; Rossi et al., 1993; Maiuri et al., 1994). However, none of these reports simultaneously described measurements of lactase and SI gene expression of the same individuals at both the mRNA and protein levels. And more importantly to the pediatric gastroenterologist, none of these studies related to the duodenum of children.

From our observations, we conclude that there are significant correlations between lactase and SI protein and their respective mRNAs, suggesting predominant gene regulation at the transcriptional level. Although statistically significant, the correlation coefficients of 0.27 and 0.33 for lactase or SI mRNA versus their respective protein levels imply additional factors to explain the variations present in our study. The existence of additional factors, regulating lactase expression in adult humans, was also recently suggested by others (Rossi et al., 1997). However, no obvious 'additional factors' are identified at present. Nevertheless, our results show for the first time, primary transcriptional control both for the lactase and SI gene in the duodenum during childhood. These results are in accordance with studies in human adults (Harvey et al., 1995; Fajardo et al., 1994; Escher et al., 1992), in vitro in the human Caco-2 cell line (Van Beers et al., 1995), but also in rat during development (Rings et al., 1994; Krasinski et al., 1994).

We found high degrees of inter-individual variation in mRNA levels among patients, even with normal mucosal morphology. This is in agreement with earlier literature measuring enzyme activity of these enzymes in adults and children (Newcomer and McGill, 1966, 1967; Olsen et al., 1996). The nature of the factors causing this large variation in expression levels are largely unknown, but the different lactase alleles (i.e. those for high and low adult lactase levels) do contain the information to direct high or low expression levels (Wang et al., 1994). It is estimated that approximately 5% of Northern Europeans are homozygous for low lactase expression during adult life, while some 35% of the population is heterozygous for this trait (Wang et al., 1994). Particularly in older children, part of the variation in expression levels of lactase may be due to this allelic variation.
Lactase and SI are expressed at stable levels in Caucasians from new-born to adult

Neither lactase nor SI mRNA levels showed significant changes during post-natal development up to adult age (18 years). This is in sharp contrast to our previous data on the expression of both glycohydrolases during post-natal development in rat. Rat lactase was very highly expressed shortly after birth, while levels declined rapidly after weaning. Rat SI was not detectable until shortly before weaning, and thereafter reached high adult levels (Rings et al., 1994; Krasinski et al., 1994). For lactase in our present study this difference can be explained by the fact that only children from Dutch Caucasian origin were analyzed, that are known to retain high levels of lactase throughout life (Flatz, 1987), in contrast to most other human races as well as all other mammalian species (Van Beers et al., 1995). Nevertheless, it is striking that also SI levels appeared stable during human post-natal development in this study.

Lactase and SI are also independently expressed at the cellular level

As indicated above, the lactase and SI genes are regulated independently, when steady state levels of the respective mRNAs are considered. Yet, this independent mode of regulation was also found at the cellular level. In virtually all individuals examples can be found, within the duodenal epithelium, of cells clearly expressing one, but not the other, protein. This is particularly true for the specific immunostaining for SI in duodenal crypts, that was found in 83% of the children studied. Lactase was never detected in the crypts by any of the four anti-lactase Mabs that were used in this study. With several independent antibodies, others have also found SI immunostaining in rat and adult human intestinal crypts (Nichols et al., 1992; Beaulieu et al., 1989). Future experiments using in situ hybridization for SI should clarify if SI protein expression is indeed supported in duodenal crypts in children.

Intracellular lactase and SI immunostaining

In 27% of the children studied, we found high intracellular levels for both lactase and SI protein that has not been reported before. We found that this staining did not correlate with sex, age, or clinical diagnosis of the patients, or with the morphology score for the biopsy sections. The localization of the staining in the enterocytes is very characteristic of the size and position of the Golgi compartment within enterocytes, which remains to be confirmed by double labelling using Golgi-specific antibodies. Per patient, the intracellular staining was always observed for both glycohydrolases simultaneously. In sections from the duodenal biopsies of these patients, the intracellular staining was found in all cells expressing lactase and SI on their brush borders, in villus as well as in crypt enterocytes, whereas staining was never detected in goblet and Paneth cells.

Intracellular staining for lactase was described before in so-called 'phenotype II' adult-type lactase deficiency (Lorenzsonn et al., 1993). This rare phenotype was observed in an adult with a mixed native American/European ancestry, and was characterized by an accumulation of immunoreactive lactase in the rough endoplasmic reticulum, while the staining for SI in this patient was abundant and only found in the brush border (Lorenzsonn et al., 1993). This phenotype seems clearly distinct from the
phenotype that we have observed here for several reasons: 1. The condition that we found was not particularly rare among ethnic Dutch individuals, 2. We observed the intracellular staining in the Golgi complex, rather than in the endoplasmic reticulum, and 3. We found intracellular staining for both enzymes, and not just for lactase.

We excluded the possibility that the intracellular staining was due to non-specific staining through a number of control experiments: 1. Results obtained using two distinct anti-human SI Mabs were indistinguishable in all cases, 2. Extraneous murine antibodies in the ascites do not contribute to the intracellular staining, since ascites obtained from a sibling mouse (containing an unrelated Mab) did not stain any of the tissue sections. Moreover, hybridoma culture supernatants containing the Mabs gave the same results as ascites, and also after protein A-purification the Mabs gave the same results, 3. The secondary antibodies used in immunohistochemistry were not responsible for the intracellular staining, and 4. Fixation artefacts were highly unlikely since alternative fixation protocols yielded a similar intracellular staining in sections of biopsies of the patients that showed this particular phenotype.

In a previous study, we had collected biopsies from two sites of the duodenum, i.e. at the bulbus duodeni and distally from the papilla of Vater (Van Beers et al., 1998). Intracellular staining, if detected in a patient, was always observed in biopsies from both sites in the duodenum. We have also studied non-Caucasian children during this earlier work, and also these patients occasionally showed intracellular staining for both glycohydrolases (Van Beers et al., 1998). These patients were not included in the present study as separate groups due to small numbers. Nevertheless, the intracellular staining phenotype seems not related to ethnic background.

It is described that particular carbohydrate epitopes, like ABO-blood group antigens, may be specifically localized to the Golgi apparatus. The intracellular staining found in this study was likely not due to recognition of carbohydrate epitopes, since the staining did not correlate to the ABO-blood group of the patients. Moreover, the anti-lactase Mab and both anti-SI Mabs that were used, were shown to be directed to peptide epitopes, since each Mab recognizes (in addition to the high-mannose and complexly N-glycosylated forms of the enzymes) the de-N-glycosylated precursor, as shown by immunoprecipitation (Van Beers et al., 1995; Hauri et al., 1985). Moreover, these Mabs did never recognize goblet cells that are known to express a wide variety of carbohydrate epitopes. Thus, at present it is very difficult to explain why this phenotype, i.e. concomitant high intracellular lactase and SI levels, occurs only in a subset of children.

**Perspectives**

We have shown in the duodenum of Caucasian children that; 1. the lactase gene is primarily regulated transcriptionally, 2. the SI gene is primarily regulated transcriptionally, 3. lactase and SI are regulated independently from each other, as detected by mRNA levels as well as at the cellular level 4, the expression levels of either lactase or SI do not change during development form newborn to adult, and 5. secondary deficiency of both glycohydrolases arises during villus atrophy.
We have additionally shown that expression levels of both enzymes vary widely in human duodenum, but seem nevertheless mainly transcriptionally regulated. However, other regulatory mechanisms may also be involved in determining variation of these expression levels. Future studies should be directed at identifying the factors that influence the lactase and SI expression levels per enterocyte as well as for individuals.

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