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Duodenal endocrine cells in mice with particular regard to age-induced changes

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Summary. Duodenal endocrine cell types in four age groups of NMRI mice (1, 3, 12 and 24 months old) were identified by immunocytochemistry and quantified by computerized image analysis. Whereas the number of secretin-immunoreactive cells was significantly increased in the 24-month-old group, the number of GIP-immunoreactive cells was reduced in 12-month-old compared with 3-month-old mice. The number of somatostatin-immunoreactive cells was fewer in both the 12- and 24-month-olds vis-à-vis the 3-month-old mice. Whereas serotonin-immunoreactive cells were fewer in both 1-month-old and 12-month-old mice, they were more numerous in 24-month-old mice then in the 3-month-old ones. The number of gastrin/CCK-immunoreactive cells was unaffected by age. The cell secretory index (CSI) of secretin- and serotonin-immunoreactive cells was increased in the 24-month-old mice vis-à-vis the 3-month-old ones and the CSI of GIP- and somatostatin-immunoreactive cells was increased in 12-month-old mice vis-à-vis 3-month-old mice. In contrast, the CSI of somatostatin- and serotonin-immunoreactive cells in 1-month-old mice was lower than that of 3-month-old-mice. The nuclear volume of secretin-, GIP-, gastrin/CCK- and serotonin-immunoreactive cells was less in 1-month-olds than in 3-month-old mice. Whereas the nuclear volume of somatostatin-immunoreactive cells was decreased in 12-month-old animals, that of gastrin/CCK- and serotonin-immunoreactive cells was greater in 24-month-old mice than in 3-month-old ones. It is concluded that these changes may be secondary to structural and functional changes in the gastrointestinal tract caused by ageing. It is possible that these changes are involved in the development of dysfunction of the gut observed at advanced age.

Key words: Ageing, Duodenum, Computerized image analysis, Immunocytochemistry, mice

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Introduction

Ageing is associated with an increased frequency of secretory and motility disorders of the gastrointestinal tract and associated glands. For example, slower gastric emptying of liquids (Moore et al., 1983), longer colonic transit time (McDougal et al., 1984; Madsen, 1992), reduced contractility of the gallbladder (Weddman et al., 1991; Ishizuka et al., 1993), and reduced pancreatic exocrine secretion (Laugier et al., 1991), have been reported in old animals and elderly humans.

The duodenum contains several endocrine cell types that secrete peptides and monoamines, which play an important role in regulation of gastrointestinal motility (Allescher and Ahmed, 1990), and secretion (Rangachari, 1990). It is therefore conceivable that the gastrointestinal disorders seen at advanced age may be associated with changes in these cells.

The purpose of this study was to establish whether the duodenal endocrine cells in a murine animal model change with advancing age. For this purpose we chose NMRI mice. These mice are randomly bred having a degree of genetic variance similar to that which would be expected in the human population.

Materials and methods

Animals

Sixty NMRI/Bom mice (Bomholtgård Breeding and Research Centre, Denmark) were used. The males and females were housed separately in cages, 5 to a cage, in a room with a 12/12 h light/dark cycle, and fed a standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) with free access to water. Ten mice (5 males, 5 females) were sacrificed when one month old, 10 more at 3 months and another 10 at 12 months. Six 24-month old males were also killed. The animals were starved overnight before being sacrificed by cervical dislocation. The proximal duodenum was excised. The investigation was approved by the local committee on animal ethics at Umeå University.

Table 1. Detailed account of the antisera used.

ANTISERA RAISED IN RABBIT AGAINST	WORKING DILUTION	SOURCE	CODE no.
Porchine secretin	1:1600	Euro-Diagnostica, Malmö, Sweden	R 787502 B33-1
Porchine gastric inhibitory polypeptide	1:800	Euro-Diagnostica	R 786403 B2
Synthetic human somatostatin	1:4000	Dakopatts, Glostrup, Denmark	A 566
Synthetic human gastrin-17*	1:1600	Dakopatts	R 783511 B36-1
Serotonin conjugated to BSA	1:1600	Euro-Diagnostica	R 871204 B 56-1

* Cross-reacts with CCK and gastrin C-terminus

Immunocytochemistry

Tissue specimens were fixed overnight in 4% buffered formaldehyde, embedded in paraffin wax and sectioned at 5 μm. The sections were immunostained with the avidin-biotin complex (ABC) method (DAKO A/S, Glostrup, Denmark) as described in detail earlier (El-Salhy et al., 1993). Briefly, the slides were immersed in 0.5% hydrogen peroxide in Tris-HCl buffer (pH 7.6) for 10 min to inhibit endogenous peroxidase activity. The sections were pre-treated with 1% bovine albumin for 10 min to occupy non-specific binding sites. Incubation with primary antisera was performed for 20 h, with the secondary biotinylated swine anti-rabbit IgG, diluted 1:200 30 min, and with avidin-biotin-peroxidase complex, diluted to 1:200, another 30 min. All incubation took place at room temperature. Peroxidase was detected by immersing the sections in 50 ml Tris-HCl buffer containing 25 mg diaminobenzidine tetrahydrochloride (DAB) and 10 μl 30% hydrogen peroxidase for 10 min followed by light counterstaining in Mayer's haematoxylin. A detailed account of the primary antisera used are presented in Table 1.

Specificity controls were the same as described previously (El-Salhy et al., 1993). In brief, they included replacement of the primary antisera with non-immune rabbit serum and preincubation of the antisera for 24 h at 4 °C with the corresponding or a structurally related antigen (75 μg/ml diluted antisera). Positive controls were obtained by immunostaining sections from the human duodenum.

Computerized image analysis

Quantification was performed using a Quantimet 500 MC Image Processing and Analysis System (Leica, Cambridge, England) linked to an Olympus microscope, type BX50. The software for this system was Leica's Windows-based analysis program, QWIN (version 1.02) and the interactive program QUIPS (version 1.02). Measurements were performed with x4, x20 and x40 objectives. At these magnifications each field on the monitor represents a tissue area of 1.3, 0.104 and 0.009 mm^2 respectively. The endocrine cells were quantified in 40 randomly chosen fields, 20 in villi and 20 in crypts from 5 sections, at least 50 μm apart. The slides were coded, mixed and the examiner did not know the age of the animals. The parameters measured were the number of immunoreactive cells, the area of epithelial cells, and also the immunoreactively stained cell area and the nuclear area of 10 nucleated cells. This was done using an automated standard sequence analysis operation, described earlier in detail (El-Salhy et al., 1997). Briefly, the immunoreactive cells were counted using field measurements. The area of the epithelial cells and that of the immunoreactively stained cell area was measured using a threshold setting. The nuclear area was measured by using field measurements where the parameter area was chosen. The data from each field were tabulated, computed and subjected to automated statistical analysis. For all these measurements a x20 objective was used, except for nuclear area, where a x40 objective was used. In order to estimate the cell content of the immunoreactive-secretory area, the cell secretory index (CSI) was used (El-Salhy et al., 1997). This was calculated as follows: CSI=VS/CN, where VS=the volume of the immunoreactive cellular area in 10 nucleated cells, and CN=the number of cells. The nuclear volume was estimated as described previously (El-Salhy et al., 1998).

The numbers of villi and crypts per mm of baseline and the height of 9 villi were measured in five fields from three perpendicularly cut sections in each mouse. The baseline was aligned with the base of the crypts. The measurements were performed using the function Field measurements where the parameter distance was chosen. These measurements were made using a x4 objective.

Statistical analysis

The 1-, 12- and 24- month-old groups were compared with the 3-month-old ones using the Mann-Whitney non-parametric U-test. p-values less than 0.05 were considered significant.

Results

Immunocytochemistry

Secretin- (Fig. 1), GIP-, gastrin/CCK-, somatostatin- and serotonin- (Fig. 2) immunoreactive cells were detected in all animals studied. Gastrin/CCK- and serotonin-immunoreactive cells were found in both villi and crypts. Secretin was found predominantly in the

villi, and GIP- and somatostatin-immunoreactive cells in the crypts. These cells varied in shape, being flask- or basket-shaped.

There was no immunostaining when the primary antisera was replaced by non-immune serum, or when it was pre-incubated with the corresponding antigen. Pre-incubation of the primary antisera with structurally related antigen had no effect on the immunostaining. The antisera stained corresponding endocrine cells in human duodenum.

Computerized image analysis

The results of the morphometric measurements are presented in Figs. 3-5. The number of secretin immunoreactive cells was significantly increased in the 24-month-old mice as compared with the 3-month-old animals. GIP-immunoreactive cells were fewer in the 12-month-old mice than in the 3-month-old group. Somatostatin immunoreactive cells were fewer in both the 12- and 24-month-old groups than in 3-month-olds. The total number of gastrin/CCK-immunoreactive cells was unaffected by age, although the number of cells was reduced in the crypts of the 12-months old mice. Whereas serotonin immunoreactive cells were fewer in both 1-month-old and 12-month-old mice, they were more numerous in 24-month-old mice than in the 3-month-olds.

The CSI of secretin- and serotonin-immunoreactive cells was higher in the 24-month-old mice than in the 3-month-olds. The CSI of GIP- and somatostatin-immunoreactive cells increased in 12-month-old mice vis-á- vis 3 month-old-mice. The CSI of somatostatin- and serotonin-immunoreactive cells was lower in 1-

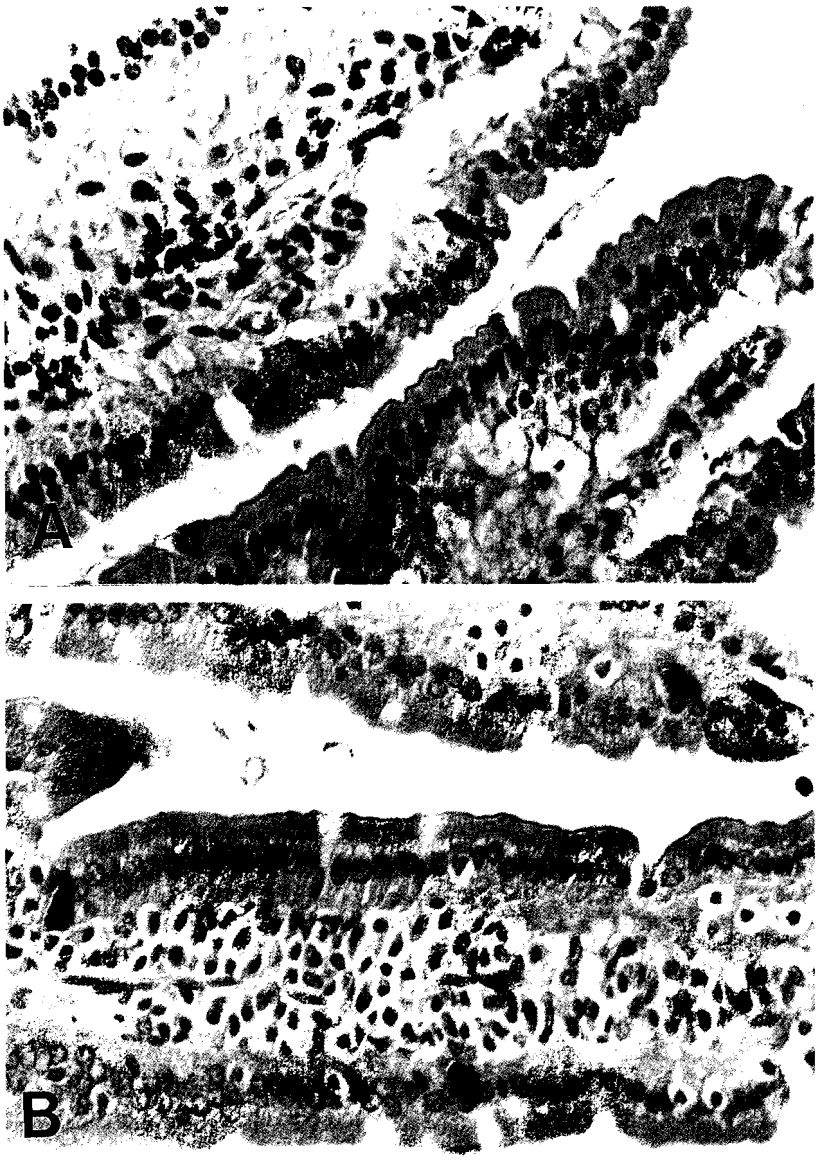

Fig. 1. Secretin-immunoreactive cells in the duodenal villi of a 3-month-old mouse **(A)** and in a 24-month-old mouse **(B)**. x 200

month-old mice than in 3-month-olds. The nuclear volume of secretin-, GIP, gastrin/CCK- and serotonin-immunoreactive cells was lower in 1-month-old than that in 3-month-old mice. Whereas the nuclear volume of somatostatin immunoreactive cells was decreased in 12-month-old mice, the nuclear volume of gastrin/CCK- and serotonin-immunoreactive cells increased in 24-month-old mice vis-à-vis 3-month-olds.

The numbers of crypts per mm in 1-, 12- and 24-month-old mice were 27±1, 44±4, 35±3 and 35±3, respectively. The corresponding figures for the villi per mm baseline were 8.9±0.3, 8.4±0.9, 8.5±0.4 and 7.8±0.4, respectively. Villous height in these age groups was 444±23, 390±24, 335±27 and 416±47 μm, respectively. All values are expressed as mean±SE. The numbers of crypts per mm base line in 1-, 12-, 24-month-old mice were significantly reduced compared with the 3-month-olds ($p<0.01$, <0.05 and <0.01, respectively). The numbers of villi per mm of baseline and their height were unaffected by age.

Table 2. The number, cell secretory index and nuclear volume of various duodenal endocrine cells (mean±SE) in males and females from different age groups of mice.

	NUMBER OF ENDOCRINE CELLS[1]		CELL SECRETORY INDEX[2]		MEAN NUCLEAR VOLUME[3]	
	male	female	male	female	male	female
Secretin						
Total	6163±853	5556±607	104±8	111±10	96±5	94±4
GIP						
Total	5635±453	4320±364	78±6	80±6	103±4	97±5
Villi	5124±426	3323±448				
Crypts	6099±616	5147±550				
Gastrin/CCK						
Total	8774±676	8809±927	146±14	124±8	104±8	115±6
Villi	8737±676	9391±1030				
Crypts	9025±919	8725±1244				
Somatostatin						
Total	4843±625	5525±573	110±13	94±4	90±5	87±5
Serotonin						
Total	29529±2558	26779±1721	161±7	157±8	115±7	114±6
Villi	21936±1581	21094±1436				
Crypts	37829±4877	32382±2327				

[1]: per mm^3 of epithelial cells, [2]: μm^3/cell, [3]: μm^3

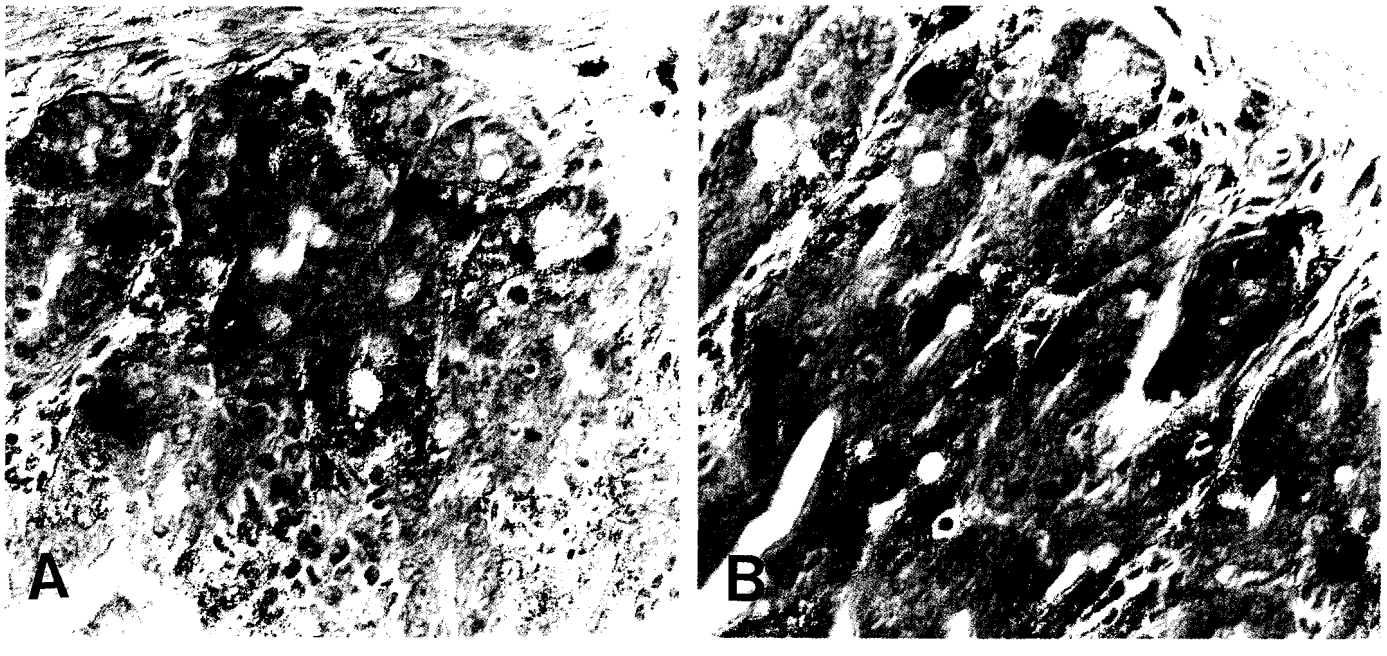

Fig. 2. Serotonin-immunoreactive cells in the duodenum of a 1-month-old mouse **(A)**, and in a 24-month-old mouse **(B)**. x 200

There was no statistical difference between males and females in any of the endocrine cell types regarding the numbers of cells, CSI, or nuclear volume (Table 2).

Discussion

The endocrine cells in the present investigation were studied by three morphometric parameters, namely the numbers of cells, CSI and nuclear volume. The former gives information about the anatomical peptide/amine-producing units. CSI indicates the immunoreactive secretory content of these anatomical units, which is a summation of the peptide/amine synthesis and its release from the cells (El-Salhy et al., 1997). The nuclear volume indicates the synthesis activity of the cell (El-Salhy et al., 1998). Thus, a large nuclear volume indicates a high peptide/amine synthesis activity and vice versa. By combining CSI and nuclear volume, the physiological activity of the peptide/amine-producing cells can be deemed. Using these parameters, both structural and physiological activity changes related to ageing were found in the murine duodenal endocrine cells.

Secretin-immunoreactive cells were significantly more numerous in 24-month-old than in 3-month-old mice. This increase was accompanied by an increased CSI, but unchanged nuclear volume, indicating that these cells had an unchanged synthesis activity, but a decreased release rate. These observations are consistent with the previously reported findings obtained by radioimmunoassay in duodenal tissue extracts from the same mice, where the secretin content was significantly higher in 24-month-old than in 3-month-old mice (El-Salhy and Sandström, 1999). In both 1- and 12-month-

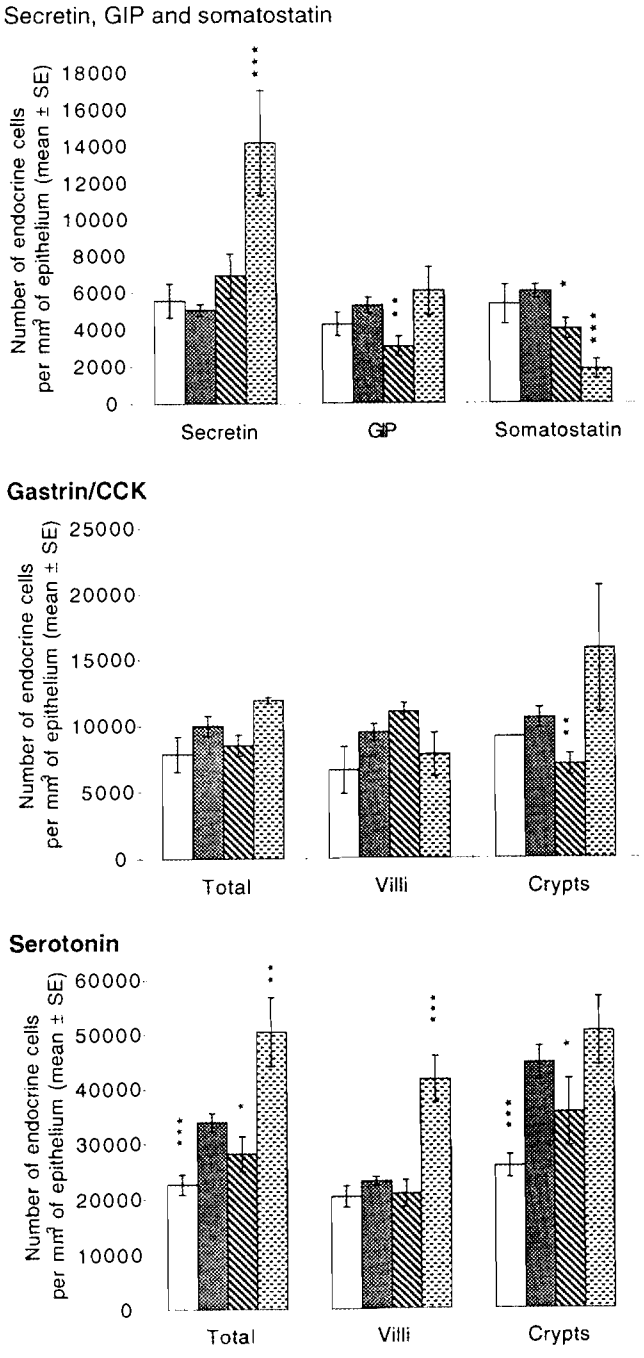

Fig. 3. The number of various endocrine cell types in different age groups. *: p<0.05; **: p<0.01; ***: p<0.001.

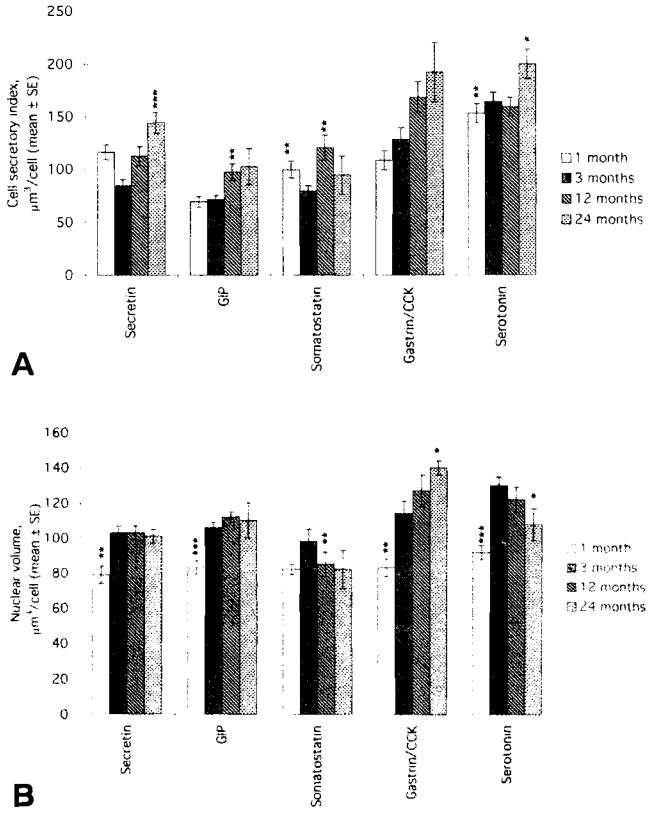

Fig. 4. The cell secretory index (**A**) and the nuclear volume of various endocrine cells in different ages (**B**). Symbols are the same as in Fig. 3.

old mice the CSI was higher than in the 3-month-old mice, though it was not statistically significant. This may account for the previously reported increased content of this peptide in duodenal extracts at these ages in the same mice (El-Salhy and Sandström, 1999). The duodenal secretin cells seem to change more in mice than in man; morphometric studies in human duodenum have not shown any age-related changes (Sandström and El-Salhy, 1999). Secretin has a trophic action on the exocrine pancreas and increases pancreatic secretion of water and bicarbonate. It increases the flow of bicarbonate and concentration of bile and inhibits basal and pentagastrin-induced gastric acid secretion (Leiter et al., 1994). Moreover, secretin inhibits the gastric emptying and contractile activity of both small and large intestine (Leiter et al., 1994). The increase in secretin may be a response to the reduced age-related pancreatic secretion (Laugier et al., 1991), and may in turn contribute to the development of the slower gastric and intestinal transit seen in advanced age.

GIP-immunoreactive cells were significantly scarcer in 12-month-old than in 3-month-old mice. It is noteworthy that GIP-immunoreactive cells occurred predominantly in the crypts and that the number of crypts in these mice was significantly reduced compared with the 3-month-old mice. It is difficult to determine whether the reduced cell number reflects an actual decrease or if it reflects a decrease in the crypts. The CSI of these cells was increased, but the nuclear volume was unchanged, indicating unchanged synthesis but a slower release rate. The present finding of fewer GIP-immunoreactive cells is in accordance with a previous observation of an age-related reduction in duodenal GIP level in NMRI mice (El-Salhy and Sandström, 1999). In 1-month-old mice the CSI was the same as that of 3-month-old mice, but the nuclear volume decreased, indicating low synthesis activity and release rate. In man, no such changes were found in duodenal GIP cells (Sandström and El-Salhy 1999).

The present findings, that somatostatin-immunoreactive cells were significantly scarcer in 12- and 24-month-old mice than in 3-month-olds are consistent with previous radioimmunoassays (El-Salhy and Sandström, 1999). As in GIP-cells, somatostatin-immunoreactive cells occurred mostly in crypts, which were fewer in both age groups with respect to the 3-month-olds. It is therefore uncertain as to whether this decrease is an actual one, or only reflects the decrease in the crypts. The CSI of somatostatin-immunoreactive cells in 12-month-old mice increased as compared with the 3-month-olds, but conversely, nuclear volume decreased, indicating low synthesis and a low release rate of these cells. It is worth mentioning that duodenal somatostatin-immunoreactive cells have been found to be more numerous in humans aged 40-49 years than in those aged 20-29 years (Sandström and El-Salhy, 1999).

As in antrum (Sandström et al., 1999) and colon (Sandström et al., 1998) of NMRI mice, the number of duodenal serotonin-immunoreactive cells increased in 24-month-old vs. 3-month-old mice. The CSI of these cells also increased, but nuclear volume decreased, indicating low synthesis and a slow release rate of this amine. On the other hand, the serotonin-immunoreactive cells were significantly fewer in 1- and 12-month-old mice than in 3-month olds. It is noteworthy that this decrease occurred in the crypts In both these age groups the crypts were fewer than in 3-month-olds. The decrease in the number of these cells in these two age groups may have been caused by this structural change rather than by an actual decrease. Serotonin has been found to stimulate pyloric contraction, and small intestinal and colonic motility, as well as to accelerate small and large intestinal transit (Goarard et al., 1994; Lindberg, 1985; Oosterbosch. et al., 1993; Tally, 1992; von der Ohe et al., 1994). The increase in the number of serotonin cells may counter-balance the decreased number of receptors, and weakened response of effector organs. In human duodenum, serotonin-immunoreactive cells have been found to be unaffected by ageing (Sandström and El-Salhy, 1999).

The present investigation of duodenal endocrine cells in a murine model showed that these cells change with age. These changes may be secondary to structural and functional changes in the gastrointestinal tract caused by ageing. It is possible that these changes are involved in the development of dysfunction of the gut observed at advanced age.

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