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RAPID COMMUNICATION

Age-related histomorphologic changes in the canine gastrointestinal tract: A histologic and immunohistologic study

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Abstract

AIM: To examine the changes in the histomorphology of the gastric, jejunal and colonic wall of dogs due to physiological aging.

METHODS: Full thickness biopsies were taken from the gastrointestinal tracts of 28 dogs of different ages. The thickness of the different layers of the wall was measured and the numbers of proliferating cells as indicated by immunohistochemical detection of Ki67 were counted.

RESULTS: In the three excision sites, the thickness of all subepithelial layers increased with rising age. The strongest correlation between age and thickness of the intestinal wall was found in the first 10 years of life and in the jejunum (r = 0.6-0.71 for the deep lamina propria mucosa, the muscularis mucosa, and the circular layer of the tunica muscularis). The number of proliferating cells decreased during aging, with the strongest correlation in the lamina propria mucosa and lamina muscularis mucosa of the jejunum and in the colonic submucosa (r = -0.61 to -0.71). Epithelial proliferation was only weakly correlated to the age.

CONCLUSION: The morphology of the deeper layers and the proliferation of mesenchymal cells of the intestinal wall of healthy dogs are correlated with age. Gastrointestinal epithelial proliferation is only weakly age-correlated.

Key words: Age; Canine; Intestine; Ki67; Stomach

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INTRODUCTION

Canine models are well known in the development of gastrointestinal drugs. On the other hand, an increasing number of biopsy samples are taken from the intestines of pet dogs with gastrointestinal symptoms and delivered to pathological diagnostic institutions for evaluation. Lesions, usually found in those specimens, however, are in most cases quantitative aberrations of normal morphology and cellular distribution rather than of a distinct qualitative kind. So a correct diagnosis is largely dependent on the experience of the examiner and the differentiation between physiological and pathological influences on the morphology. Data on the histological changes in the intestinal morphology in dependence on physiological processes, however, are very sparse. Suckling puppies exhibit rapid changes in the mucosal morphology that reach a comparatively stable state by the 42nd d of life[1]. In old dogs, Lafora body-like polyglucosan bodies have been described in the smooth musculature[2] and amyloid in the vessel walls of the large and small intestine without functional or neurological abnormalities[3]. Because of the usage of endoscopic biopsies, however, most examinations are limited to the mucosa[4]. The aim of this study was to characterize morphological alterations during the physiological course of aging including the different layers of the intestinal and gastric wall of dogs.

MATERIALS AND METHODS

Subjects

Tissue samples were obtained from 28 dogs of different age and breed (Table 1), which were patients of the Small Animal Clinic and were euthanatized for diseases unrelated to the gastrointestinal tract. Under general anesthesia, full thickness biopsies of the gastric, jejunal and colonic wall were surgically excised and immediately fixed in 100 mL/L

Table 1 Distribution of age, gender and breed

Age (yr)	Gender	Breed
0.25	M	Collie
0.5	M	Boxer
0.75	F	Rottweiler
0.75	M	West Highland White Terrier
2	F	Newfoundland
3	M	American Canadian White Shepard
3	M	Doberman-Pinscher
5	M	Fox Terrier
5	M	Mongrel
6	M	Pommeranian
6	F	German Shepherd Dog
7	M	Bernese Mountain Dog
7	F	Schnauzer
7	F	Caucasian Shepherd
7	F	Rottweiler
8	M	Mongrel
8	M	German Shorthaired Pointer
8.5	F	Mongrel
9	F	Mongrel
10	M	Mongrel
12	F	English Setter
12	M	Mongrel
12	M	Gordon Setter
13	F	Hovawart
13	F	Mongrel
14	F	Mongrel
14	F	Rottweiler
17	F	Mongrel

M: Male; F: Female.

neutral formalin. After fixation, the samples were routinely embedded in paraffin, cut, and stained with hematoxylin and eosin.

Evaluation of slides

All measurements were performed in a blind fashion, i.e., without knowledge of the animals' age. Distances and structural measurements were performed using a computer sustained manual system (ASM 68k), in which a line was projected into the microscopic ocular, that could be manually adjusted to the outlines of the respective structure to be determined. The resulting length of the line was calculated by the supporting computer system.

The following parameters were determined: in the stomach, depth of foveolae (epithelial length from the surface to the isthmus), length of whole gastric glands (epithelial length from the surface to the base of the gland), estimated percentage of chief cells and parietal cells in the pars principalis, number of fiber layers between the gland base and the lamina muscularis mucosa, thickness of the lamina muscularis mucosa, submucosa, and, because of the inconsistent orientation and borders of the single muscle layers in the stomach, of the tunica muscularis as a whole (Table 2); in the jejunum, villus length (length from the tip following the central lymphatic vessel to the crypt mouth), villus mid and base width (shortest distance from epithelial surface to epithelial surface in the middle of the villus and at the crypt mouth, respectively), crypt depth (length from the crypt mouth to the crypt base following the crypt lumen), thickness of the stratum compactum (distance between crypt base and lamina muscularis mucosa), lamina muscularis mucosa, submucosa, circular and longitudinal layer of the tunica muscularis mucosae (Table 3); in the colon, crypt depth, thickness of the lamina muscularis mucosa, submucosa, circular and longitudinal layer of the tunica muscularis mucosa (Table 4). All measurements were performed at four points of a well oriented section, which were equally distributed over its total length.

Table 2 Absolute values in the stomach

Age (yr)	Depth of foveolae (μm)	Glandular length (μm)	Chief cells (%)	Fibre layers (*n*)	Muscularis mucosa (μm)	Submucosa (μm)	Tunica muscularis (μm)
0.5	210	952	40	6	89.8	60	2076
0.75	226	1073	40	3	99.3	1157	2341
0.75	146	1076	60	4	146.8	365	1746
2	152	1249	70	6	133.0	866	2526
3	155	795	50	11	96.8		
3	228	1052	0	4	212.5	341	1600
5	169	942	70	4	142.3	867	2221
5	198	1013	60	5	96.5	972	1652
7	139	1012	40	3	140.0	869	3140
8	204	927	70	2	192.8	524	1488
8	141	774	30	7	136.8	653	2684
8	219	1074	30	4	154.8	169	968
9	167	986	60	4	249.0	551	2974
10	220	1146	40	6	122.8	1075	2337
12	245	1017	50	9	178.8	3501	2875
12	166	1165	40	5	112.8		
12	230	1059	70	6	121.3	1102	2191
14	189	795	20	12	154.5	1250	1205
17	294	815	20	6	120.5	694	2038

Immunohistochemical assay

In the jejunum and colon, where the most obvious changes in the thickness of wall layers were found, immunohistochemical detection of Ki67 was carried out to determine the proliferative index. The immunohistochemical reaction was performed as follows: Paraffin-embedded sections were dewaxed and treated with 50 mL/L H_2O_2 in ethanol for 30 min to inhibit endogenous peroxidase, followed by rinsing three times in phosphate-buffered saline (PBS). Antigen-retrieval was achieved by microwave treatment (20 min, 0.01 mol/L citrate buffer, pH 6). After demasking, the slides were incubated overnight with the first antibody (MIB-1, DAKOCytomation, Glostrup, Denmark) diluted 1:100 in PBS with 10 g/L bovine serum albumin. Binding of primary antibody was detected with a biotinylated second antibody (goat anti-mouse, diluted 1:200 in PBS) and the ABC-reagent (both Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions using diaminobenzidine as chromogen. Finally, the slides were counterstained with hematoxylin. Negative control was performed with an identical procedure without the first antibody in the PBS/serum incubation step. An internal

Table 3 Absolute values in the jejunum (μm)

Age (yr)	Villus length	Crypt depth	Stratum compactum	Muscularis mucosa	Submucosa	Circular tunica muscularis	Longitudinal tunica muscularis
0.25	572	448	17	58	92	443	254
0.5	622	354	30.75	52	327	573	387
0.75	981	1051	24.25	82	273	729	478
0.75	570	269	35	56	193	519	304
2	536	435	39.75	88	161	542	182
5	894	412	67.25	67	229	764	368
5	882	303	37.75	62	183	658	338
6	867	406	62.75	68	217	585	249
7	589	623	45	73	176	602	346
7	906	466	32.25	99	228	766	454
7	630	381	56.25	106	292	639	418
8	596	333	87.25	162	232	802	285
12	647	314	108.25	150	294	1152	446
12	653	426	46.75	59	161	579	334
13	703	506	73	112	364	1047	217
13	709	344	53.4	146	339	672	290
14	726	475	44.75	89	267	793	370
14	700	514	104.2	136	233	971	535
17	695	403	40.5	75	309	693	304

Table 4 Absolute values in the colon (μm)

Age (yr)	Crypt length	Muscularis mucosa	Submucosa	Circular tunica muscularis	Longitudinal tunica muscularis
0.25	520	41.3	122	387	1235
0.5	530	40.3	165	454	707
0.75	488	60.0	203	338	537
0.75	515	52.8	133	540	530
2	515	56.3	362	541	562
5	609	54.0	388	557	968
5	577	82.3	349	792	682
6	533	48.3	531	728	701
6	363	51.0	375	449	855
7	716	77.7	299	617	1307
7	443	63.0	165	534	586
7	404	38.1	146	519	553
8.5	343	73.0	162	812	787
10	567	44.5	223	734	1034
12	485	40.5	462	510	568
12	460	59.5	372	641	810
13	399	45.5	191	776	1780
13	612	72.5	201	810	981
14	481	37.8	1397	784	575
17	607	48	315	607	359

positive control for the proliferation marker was present in all sections in the epithelial renewal zone of crypts and gastric pits.

Evaluation of Ki67-positive cells

In the jejunum and colon, the number of Ki67-positive cells (i.e. proliferating cells) was examined for the epithelia. Myocytes, myofibrocytes and fibrocytes could not be properly differentiated by morphological means in the immunohistochemically stained slides. For this reason, the number of proliferating cells per area comprises all cells of the lamina propria mucosa, lamina muscularis mucosa, submucosa, circular and longitudinal layer of the tunica muscularis, except round cells, especially leucocytes. All epithelial cells of approximately the lower 40% of the crypts stained positive for Ki67. Hence, comparison was made between the relative and absolute length of this compartment of the crypts. For the other layers, the absolute numbers of positive cells were counted in one whole section and set in relation to the total area of this layer in the respective section (Tables 5 and 6).

Statistical analysis

Values were checked for normal distribution. r is the Pearson product moment correlation coefficient as calculated by OpenOffice.org Calc.

RESULTS

General considerations

In the examined sections, no qualitative changes, such as scars or amyloid deposits, were found irrespective of the age of the dogs.

Stomach: In the fundic mucosa, only minor changes could be noted with increasing age. A tendency towards a relatively increased depth of foveolae could be noted and the lamina muscularis mucosa became thicker (Table 7). The relationship between age and lamina muscularis mucosa thickness was highest in dogs younger than 10 years (n = 14).

Jejunum: The mean thickness of all layers of the jejunum increased in size during aging (data not shown). The most significant changes were detected in the deeper layers of the intestinal wall during the first 10 years of life (n = 12), most obviously in the deepest part of the mucosal lamina propria in the jejunum. The distance between the crypt base and the lamina muscularis mucosa showed a strong correlation with the age of the examined dogs (Table 8). While villus length and crypt depth did not show any clear relationship with the age, the whole mucosal thickness increased with aging, mainly in the first 10 years of life. In the lamina muscularis mucosa, submucosa and circular layer of the tunica muscularis, the thickness also increased with age. In addition, in the lamina muscularis mucosa and circular layer, these changes displayed a stronger relationship in dogs less than 10 years. The longitudinal layer of the tunica muscularis did not show age-related changes.

Colon: The circular layer of the tunica muscularis displayed an age-related increase (Table 9). In contrast to the findings in the jejunum, only weak correlations between age and the thickness of the other mucosal or submucosal layers could be found.

Immunohistochemistry

Jejunum: In all layers of the intestinal wall, the numbers of proliferating cells were highest in young dogs, particularly those less than 3 years (data not shown). A

Table 5 Proliferation in the jejunum

Age (yr)	Proliferative length epithelium		Proliferating cells (*n*)					
	Absolute (μm)	Relative (%)	Villous lamina propria (/villus)	Intercrypt lamina propria (/intercrypt space)	Lamina propria below crypts	Muscularis mucosa (/mm length)	Submucosa (/mm length)	Tunica muscularis (/mm length)
0.25	296.4	69.3	4.57	0.77	22.62	8.25	6.25	1.28
0.5	179.2	75.2	25.21	2.16	35.50	10.50	1.34	0.37
0.75	278.4	61.5	3.79	1.22	14.39	5.19	2.99	0.58
0.75	1006.4	77.0	1.23	0.43	31.07	0.00	0.00	0.06
2	151.2	47.6	1.11	0.29	2.89	0.00	0.00	0.00
5	277.6	70.3	7.48	1.16	5.56	4.17	1.64	0.33
5	314.4	73.1	9.53	0.68	17.84	1.66	1.14	0.21
6	243.8	76.8	3.24	0.00	1.06	0.98	0.00	0.24
7	378.6	71.6	2.25	0.24	8.82	0.96	0.00	0.00
7	388.0	73.2	1.61	0.47	4.66	1.49	0.18	0.00
7	412.4	70.3	7.81	0.00	4.04	0.00	1.04	0.20
8	219.6	57.7	2.89	0.00	2.86	0.39	0.00	0.00
12	139.0	60.8	4.43	0.31	13.20	0.00	0.00	0.00
12	358.0	77.6	6.78	1.33	15.14	0.00	1.65	0.20
13	231.6	77.4	16.18	1.03	19.14	0.91	0.26	0.93
13	291.4	66.8	1.38	0.00	0.00	0.00	0.00	0.00
14	335.2	73.0	2.42	0.29	1.67	0.64	1.12	0.31
14	369.4	77.1	15.55	0.75	15.04	2.70	0.18	0.09
17	248.0	65.8	0.81	0.23	2.65	0.00	0.00	0.00

Table 6 Proliferation in the colon

Age (yr)	Proliferative length epithelium		Proliferating cells (*n*)				
	Absolute (μm)	Relative (%)	Intercrypt lamina propria (/intercrypt space)	Lamina propria below crypts	Muscularis mucosa (/mm length)	Submucosa (/mm length)	Tunica muscularis (/mm length)
0.25	147.4	28.8	2.117	1.59	32.07	5.21	1.33
0.5	179.0	32.2	0.943	1.67	9.20	2.63	0.12
0.75	90.2	20.3	0.388	0.36	0.98	0.78	0.04
0.75	217.8	39.4	2.048	0.79	1.64	1.21	0.09
2	129.8	27.8	0.194	0.28	0.00	0.39	0.00
5	192.0	32.9	2.251	0.89	3.02	1.14	0.17
5	219.5	36.9	0.657	0.71	2.63	0.37	0.11
6	189.2	56.4	0.276	0.11	1.05	0.43	0.59
6	194.4	37.4	0.563	0.33	1.35	0.62	0.10
7	118.4	29.8	0.248	0.00	0.00	0.40	0.00
7	158.0	31.8	0.226	0.18	0.00	1.12	0.05
7	215.8	33.3	0.838	0.63	2.01	0.52	0.00
8.5	150.0	37.1	0.583	0.24	1.12	0.00	0.05
10	132.8	26.1	1.059	1.60	3.18	0.21	0.04
12	151.8	32.8	1.031	1.49	6.13	0.27	0.16
12	173.4	33.9	0.6519	0.55	1.84	0.29	0.00
13	132.8	26.1	0.000	0.33	1.79	0.85	0.20
13	163.6	25.5	0.817	0.29	0.66	0.24	0.02
14	138.2	30.9	0.831	0.37	3.27	0.00	0.00
17	234.0	39.4	0.329	0.40	0.00	0.18	0.00

marked correlation between age and number of Ki67-positive cells could be found in dogs younger than 10 years (n = 12) for the lamina propria between crypts and between lamina muscularis mucosa and crypt base, for the lamina muscularis mucosa, for the submucosa and for the tunica muscularis (Table 9). Including the older dogs (> 10 years), correlation between age and thickness of the lamina muscularis mucosa remained strong. In contrast to the mesenchymal tissues, no clear correlation could be detected between age and epithelial proliferation.

Colon: As in the jejunum, the number of proliferating cells decreased with age. The strongest correlations were found, again, until the age of 10 (n = 13). In particular, the thickness of the lamina propria between crypt base and lamina muscularis mucosa, of the lamina muscularis mucosa and of the submucosa revealed a strong age-

Table 7 Strength of correlation between age and diameter of different layers in the stomach wall of dogs

	All ages	< 10 yr
Absolute foveolar depth	0.44	0.05
Relative foveolar depth	0.54	0.11
Lamina muscularis mucosa	0.17	0.58
Submucosa	0.35	0.17
Tunica muscularis	-0.04	0.01

Table 8 Strength of correlation between age and diameter of different layers in the jejunal wall of dogs

	All ages	< 10 yr
Villus length	-0.01	0.17
Crypt depth	-0.15	-0.2
Whole mucosa	0.5	0.1
Lamina propria	0.54	0.72
Lamina muscularis mucosa	0.51	0.61
Submucosa	0.46	0.13
Circular muscle layer	0.59	0.6
Longitudinal muscle layer	0.12	0.12

Table 9 Strength of correlation between age and diameter of different layers in the colonic wall of dogs

	All ages	< 10 yr
Crypt depth	0.01	-0.21
Lamina muscularis mucosa	-0.05	0.42
Submucosa	0.38	0.27
Circular muscle layer	0.61	0.63
Longitudinal muscle layer	0.2	0.17

Table 10 Strength of correlation between age and number of proliferating cells in the different wall layers of the jejunum and colon

	Jejunum		Colon	
	All ages	< 10 yr	All ages	< 10 yr
Absolute length of proliferating epithelium	-0.21	-0.16	0.07	0.17
Relative length of proliferating epithelium	0.15	0.07	0.02	0.37
Lamina propria of villi	-0.07	-0.28		
Lamina propria between crypts	-0.32	-0.62	-0.36	-0.43
Lamina propria below crypts	-0.46	-0.74	-0.24	-0.65
Lamina muscularis mucosa	-0.56	-0.61	-0.36	-0.5
Submucosa	-0.45	-0.54	-0.57	-0.61
Tunica muscularis	-0.29	-0.56	-0.36	-0.34

dependency in the number of proliferating cells (Table 10). In the submucosa, the correlation remained strong throughout the lifetime. The tunica muscularis and lamina propria between crypts showed only moderately age-related proliferation (r = -0.34 and -0.43, respectively).

DISCUSSION

The layers of the gastrointestinal wall beneath the mucosa comprise the major part of intestinal tissue. However, apart from pathological conditions, these tissues are thought to remain unchanged throughout lifetime. This study revealed that in dogs without gastrointestinal diseases, a continuous thickening of the deeper intestinal layers occurs, mainly during the first 10 years of life. In dogs, this period is regarded as young to middle-aged. Hence senile change in nutritional behavior and metabolism is unlikely to be the primary driving force in this development.

The strongest correlation between age and morphology could be detected in the jejunal lamina propria mucosa between crypt base and lamina muscularis mucosa. In textbooks of veterinary histology, this site is often referred to as stratum compactum[5], when it is easily discernable from the overlying lamina propria, as in the proximal small intestine. In the examined sections, no morphologically distinct stratum compactum could be detected, even in those dogs that presented with a very large distance between the crypt base and the lamina muscularis mucosa. This might be due to the more distally located excision site. This layer is thought to prevent perforation of the intestinal wall by food containing bones[4]. Similarly, the observed proliferation of this zone during lifetime in this study might have been stimulated by mucosal distension and alteration by sharp bone fragments. However, detailed anamnestic data about the diet fed to these dogs during their whole lifetime were not available.

Observations of alterations in the size of the lamina muscularis mucosa are restricted to experimental conditions, e.g. an increase in thickness after partial jejunectomy[6] in rats. The observed increase in the thickness of the muscular layer during lifetime is paralleled by observations in the jejuna of rats[7,8]. A possible explanation might be an increased workload for the musculature caused by a decrease in neuronal coordination as a result of the neuronal cell loss during aging, which has been described in humans[9] and rats[10]. If the increased muscular thickness is the result of a hypertrophy of muscle fibers or of the proliferation of connective tissue between fibers needs further, preferentially electron microscopic investigations.

In rats, epithelial cell proliferation increased in the first postnatal week and decreased or remained unaltered in the following months[7,11,12]. Differentiation of the brush border enzymes follows a similar course with a maximal development in young adulthood[13]. Examination of the mechanisms involved pointed to involvement of proliferation, apoptosis and crypt fission in the development of the intestinal mucosal epithelium[14,15], but delivered contradictory results in particular with regard to alteration of the apoptotic rate in aging rats[14,16-18]. In contrast to these findings, the epithelial proliferation in the canine tissues in this study and a study of the jejunal mucosal morphology in dogs of different weights and ages[19] remained stable throughout lifetime. This observation was also made in mice[20]. The discrepancy in the proliferative rate might be explained by different methods applied. While bromodeoxyuridine[16] and Ki-67

(this investigation) cover a small time span of the cycling process, proliferating cell nuclear antigen is expressed during a long period exceeding mitosis. The increased number of proliferative cells in aging rats found in the study using the latter method[18] might indicate rather decreased degradation of the detected enzyme than increased number of cycling cells. The deeper layers of the present study, however, contained a decreasing number of proliferating cells with rising age. This reduced speed of cellular turn-over might lead to a lower adaptability towards a changed digestibility of diet and a slower healing after damage in older dogs.

In conclusion, all layers of the canine jejunal and colonic wall, except for the epithelial monolayer, increase in thickness during aging, while the number of proliferating cells decreases. The underlying mechanisms and possible functional consequences need further investigation.

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