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Regulation of apoptotic cell death in the pyloric glands of the canine stomach

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Abstract In gastrointestinal epithelia, apoptosis has been thought to play a part in the shedding of postmitotic cells into the lumen. However, we have found that apoptosis more frequently in the generative cell (G) zone (the lower one third of the pit) than in the luminal zone (the upper one third of the pit) and the gland zone in the canine pyloric gland. To analyse the regulation of apoptotic cell death in each zone, we labelled S-phase cells by single and repeated injections of bromodeoxyuridine (BrdU) IV at intervals of 8 h. We found that 30% of apoptoses in the G zone were flash-labelled by BrdU and might derive from cells in or just after the S phase. The incidence of apoptosis and mitotic index did not change significantly after repeated injections of BrdU until the 49-h point, when the incidence of apoptosis increased and the mitotic index decreased significantly in the G zone, while the incidence of apoptosis decreased in the luminal zone. The BrdU-induced increase of apoptosis and cell-cycle arrest at the 49-h point may be caused by enhanced DNA mispairs that are elicited by incorporation of BrdU, in particular using the template of BrdU incorporated DNA. Apoptosis in the luminal zone may be down-regulated by reduced cell production in the G-zone.

Key words Apoptosis · Bromodeoxyuridine (BrdU) · Stomach · Generative cell zone · Dog

Introduction

Gastrointestinal glandular epithelia, in which regular cell proliferation and migration are known to occur [8, 14, 16, 19], give us a simple system suitable for the study of the kinetics of the occurrence of apoptosis. In the intestine of rodents, kinetic data on the occurrence of apoptosis and radiation-induced apoptosis have been accumulated [1, 4, 15]. In small intestine and colon, apoptosis has been reported to occur at the tip of villi and in the uppermost part of the crypt, which is exposed to the mucosal surface [7]. It serves as a removal mechanism for mature cells in a tissue kinetic system with rapid cellular turnover. In the stomach, however, there are few quantitative data on the relationship between cell proliferation and the occurrence of apoptosis, because both the incidence of apoptosis and its response to irradiation are much smaller in the rodent stomach.

We have found that apoptosis in the glandular epithelium is much more frequently detected in the canine than in the rodent stomach. Using single and intermittent injections of bromodeoxyuridine (BrdU), we studied the kinetics of the occurrence of apoptosis in various parts of the canine gastric gland in the steady state and in a state of heavy BrdU administration.

It is known that administration of excessive amount of thymidine and thymidine analogues including BrdU results not only in cell cycle arrest at G_2 phase (through the imbalance in the cellular pool of deoxyribonucleotide precursors), but also in DNA strand breaks [3, 18]. In a condition of continuous exposure of cells to a fairly low concentration of BrdU in vitro, apoptotic cell death is reported to occur in a time-dependent manner [13]. The drawback of such a system, however, is the impossibility of discriminating between the effect of free BrdU on DNA precursor metabolism and the effect of the BrdU incorporated into DNA strands. In order to analyse the latter effect (DNA mispairs), we adopted intermittent instead of continuous exposure to BrdU; the imbalance of deoxyribonucleotide precursors is expected to recover soon after each injection of BrdU. This is because the extracellular BrdU is rapidly released to the urine in vivo and the intracellular BrdU in the thymidine pool may be rapidly washed out owing to rapid turnover [5].

Using this labelling protocol, we demonstrated that incidence of apoptosis in proliferative cells, which was

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higher than that of mature postmitotic cells, was further increased by intermittent BrdU injections – probably through the generation of DNA mispairs and strand breaks. Regulation of apoptotic cell death of mature cells was linked to cell proliferative activity of the generative cell (G) zone in the pyloric gland of the canine stomach.

Materials and methods

Eighteen 1-year-old and six 3- to 5-year-old beagle dogs were used: 8 were given a single injection of BrdU (100 mg/kg body weight IV) about 1 h before sacrifice (flash label); 6 and 5 dogs were given 4 and 7 repeated injections of BrdU (each of 100 mg/kg body weight IV) at 8-h intervals, respectively. (The interval of 8 h corresponds to the duration of DNA synthetic phase.) The animals' stomachs were removed 1 h after the last injection. The other 5 were control dogs and received no BrdU injections.

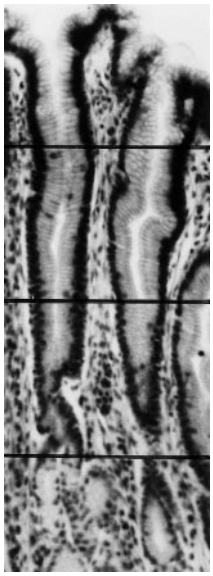
The stomachs were fixed in 10% formalin and embedded in paraffin. From 2 or 3 blocks of the pyloric region, serial sections were made for immunohistochemical detection of BrdU by the avidin-biotin-peroxidase complex method and detection of apoptosis by the TdT-mediated dUTP-biotin nick end labelling (TUNEL) method as well as for histological examination.

Part of the pyloric mucosa was taken while fresh for an electron microscopic study of apoptosis in 3 of the dogs. The sampled tissues were fixed in 2% glutar-/2% paraform-aldehyde in 0.05 M cacodylate buffer (pH 7.4) at 4°C overnight, postfixed in 1% osmium tetraoxide for 1 h at room temperature and embedded in epoxy resin (Quetol 812, Nissin EM, Tokyo). After staining of the ultrathin sections with uranyl acetate and lead citrate, electron micrographs were taken with an electron microscope (Hitachi H-500).

We detected BrdU immunoreactivity following the method described previously [22, 23] with modifications. Briefly, before applying anti-BrdU antibody, we pretreated sections in 1 N HCl, 0.05% proteinase (type XXIV, Sigma, St. Louis, Mo.) and again in 1 N HCl for 20 min each. Incubation with monoclonal mouse anti-BrdU (Becton-Dickinson, Mountain View, Calif., 1:50) was done at 4°C overnight, followed by incubation with biotinylated goat anti-mouse antibody and streptavidin-biotin-peroxidase complex (Histofine SAB-PO(M) kit, Nichirei, Tokyo) for 30 min each. The nuclei were lightly counterstained with haematoxylin.

To detect DNA strand breaks, TUNEL was carried out according to the method of Gavrieli et al. [7] after microwave irradiation [21]. We used the Mebstain Apoptosis Kit (MBL, Nagoya). Briefly, after dewaxing and rehydration, the tissues were immersed in 10 mM citrate buffer (pH 6.0) and placed in a microwave oven (Hitachi, MR-M33, Tokyo) at 500 W for 2 min. Subsequently, slides were gently cooled in a bath of cold tapwater and transferred to PBS. The irradiated sections were digested with 20 μg/ml proteinase K in PBS for 10 min at room temperature. Then, 100 μl of TdT solution (mixture of 90 ml of TdT buffer, 5 μl of biotinylated dUTP and 5 μl of TdT), containing 0.3 U/μl TdT and 0.04 nmol/μl biotinylated dUTP, were added to cover the sections, which were then incubated in a moist chamber for 60 min at 37°C. Sections were washed with TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature, and then with PBS. They were subsequently incubated with peroxidase-labelled streptavidin for 30 min and finally stained with diaminobenzidine–H_2O_2 solution. Sections were lightly counterstained with haematoxylin.

We counted apoptosis using a 40× objective lens in 50 pyloric glands selected to include the full depth of foveolar pit in the sections. Apoptotic cells were recognized as contracted cells with condensed nodular, ring-like or beaded nuclear chromatin [24] that were positively stained by TUNEL method. Apoptotic bodies consisted of small, rounded, dense chromatin fragments. As the apoptotic cells and bodies were often difficult to differentiate at the light microscopic level, we use the term apoptoses to represent both apoptotic cells and bodies. We divided the pyloric gland into four portions: the luminal zone (upper one third of foveolar pit), the transitional zone (middle one third of foveolar pit), the G zone (lower one third of the foveolar pit) and the gland zone (Fig. 1). In each zone, we calculated the incidence of apoptosis per 50 glands. We counted labelled apoptosis using a 100× objective lens and calculated the percentage of BrdU-positive fraction of the apoptosis in each zone. Similarly, the mitotic index of each zone was determined as the total number of mitosis in 50 pyloric glands. Statistical assessment of the data was done by Student's T-test, and the difference was considered to be significant when the *P*-value was smaller than 0.05.



Position in a gland	Non-labelled	Period of BrDU labelling		
		1 h	25 h	49 h
Luminal zone	*n*=5 20.00± 4.47a	*n*=8 17.00± 4.04	*n*=6 17.17± 4.71	*n*=5 11.00± 2.65a′
Transition zone	2.00± 0.71b	1.88± 1.36	3.33± 1.97	11.40± 5.68b′
G-zone	28.20± 16.50c	30.50± 16.84 (30.22± 14.41)	46.67± 12.20c″ (67.16± 13.10)	66.60± 3.85c′ (91.88± 2.55)
Gland zone	4.67± 1.53	3.88± 1.25	3.83± 0.75	4.40± 0.89

Fig. 1 Incidence of apoptosis (mean±SD) per 50 gastric glands (*n* number of dogs). Numbers in brackets indicate percentages of all apoptoses that were BrdU labelled. *a–a′ P*=0.007, *b–b′ P*=0.02, *c–c′ P*=0.005, *c–c″* not significant (*P*=0.08)

Results

In our staining conditions, TUNEL-positive cells were mostly restricted to apoptotic cells with condensed chromatin. In the pyloric glands of the dogs that received no BrdU administration, some TUNEL-positive cells were encountered in the luminal zone (Fig. 2a), more frequently in the G zone (Fig. 2b) and rarely in the transition and the gland zones.

Figure 3 demonstrates the ultrastructure of a typical apoptotic cell in the G zone of the gastric pit. Such a cell is typically located on the basal side of the epithelium (Fig. 3a). The nucleus is markedly electron dense and the cytoplasm is condensed (Fig. 3b), with mitochondria (arrows in Fig. 3c). These pictures conform to those of classic apoptosis. The presence of mucous granules in the cytoplasm (arrowheads in Fig. 3c) indicates the epithelial origin of the cell.

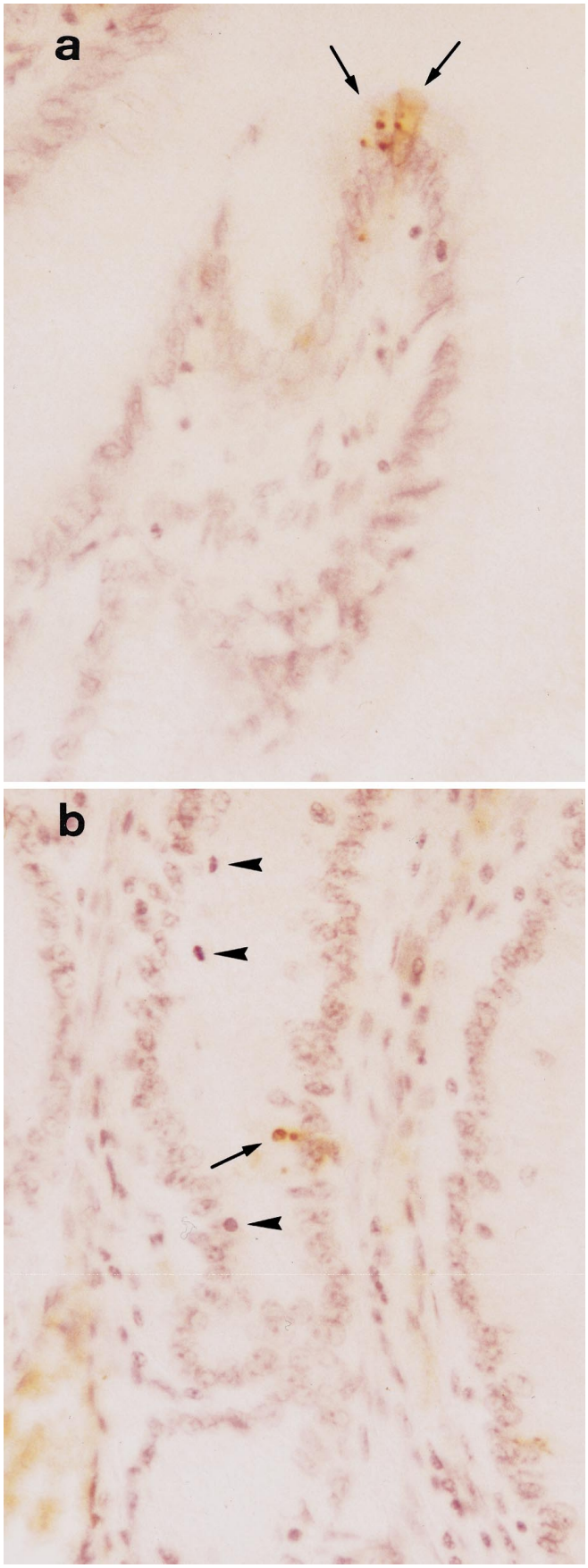


Table 1 Mitotic index (mean±SD) per 50 gastric glands (*n* number of dogs)

Position in a gland	Nonlabelled	Period of BrdU labelling		
		1 h	25 h	49 h
	n=5	*n*=8	*n*=6	*n*=5
Luminal	0	0	0	0
Transition	0	0	0	0
G-zone	29.2±10.3*	32.6±12.1	31.7±6.0	13.8±4.8*
Gland zone	0	0	0	0

P=0.0025

The incidence of apoptosis and mitosis did not change significantly from the level in non-labelled dogs following single or repeated injections of BrdU for up to 25 h in either zone (Figs. 1, 4, 5). After intermittent labelling for 49 h, the incidence of apoptosis increased in the G-zone (Fig. 6) and the transition zone, while it decreased in the luminal zone and showed no significant change in the gland zone (Fig. 1).

After 49 h of intermittent labelling with BrdU, the labelled zone was expanded to the transition and the gland zones, but the mitotic index of the G-zone reduced significantly (Table 1). Mitotic figures were hardly seen in the labelled cells in the transition and gland zones. This discrepancy indicated that the labelled cells were largely postmitotic cells even at the 49-h point.

Apoptoses were scattered at random in the labelled zone. In the G-zone, 30.3%, 67.2% and 91.9% of apoptoses were labelled by single and intermittent injections for 25 h and 49 h, respectively (Figs. 4–6). In the luminal zone, labelled apoptoses were hardly seen even after 49 h of intermittent labelling.

At the 49 h point of intermittent labelling, the increased number of apoptoses in the transition zone was largely labelled (77.3±9.1%). In the gland zone, the labelling index of apoptosis increased markedly to 66.7±24.9%, as against 7.5±11.7% at 25 h (*P*=0.004). At least some of the labelled apoptoses in these zones may have migrated from the G zone. The non-labelled apoptoses decreased not only in the luminal zone, but also in the gland zone; the incidence of non-labelled apoptoses in the gland zone (per 50 glands) decreased from 3.9±1.3 at 1 h to 1.4±1.0 at 49 h (*P*=0.002). In the gland zone, this reduction in non-labelled apoptotses and the above-mentioned increase in labelled cells were balanced, so that the frequency of total apoptosis was not significantly changed. In the foveolar pit these two changes occurred separately in the luminal and the transition zones.

Fig. 2 TUNEL-positive apoptoses in **a** the luminal zone and **b** the G-zone of a non-labelled dog. The *arrows* indicate apoptoses. The *arrowheads* indicate mitotic figures. ×1000

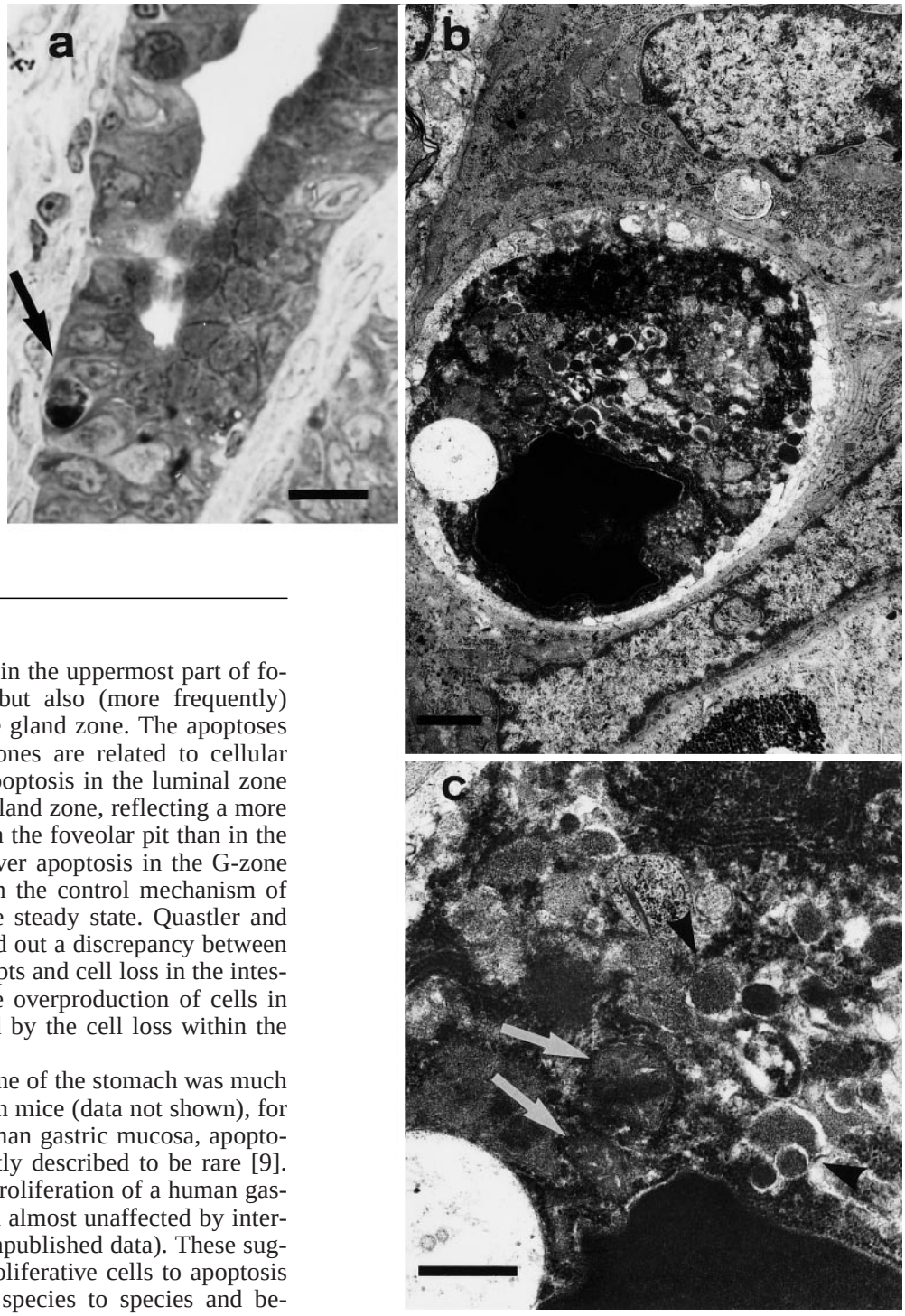


Fig. 3a–c Ultrastructure of an apoptosis in the G zone. **a** Semithin section of the G zone of the pit. The *arrow* indicates an apoptosis located on the basal side. *Bar* 10 μm **b** Electron micrograph of the apoptosis indicated by the *arrow* in **a**. It has an eccentric, condensed nucleus. *Bar* 1 μm **c** Higher magnification of **b**. The condensed cytoplasm contains mitochondria (*arrows*) and mucous granules (*arrowheads*). *Bar* 0.5 μm

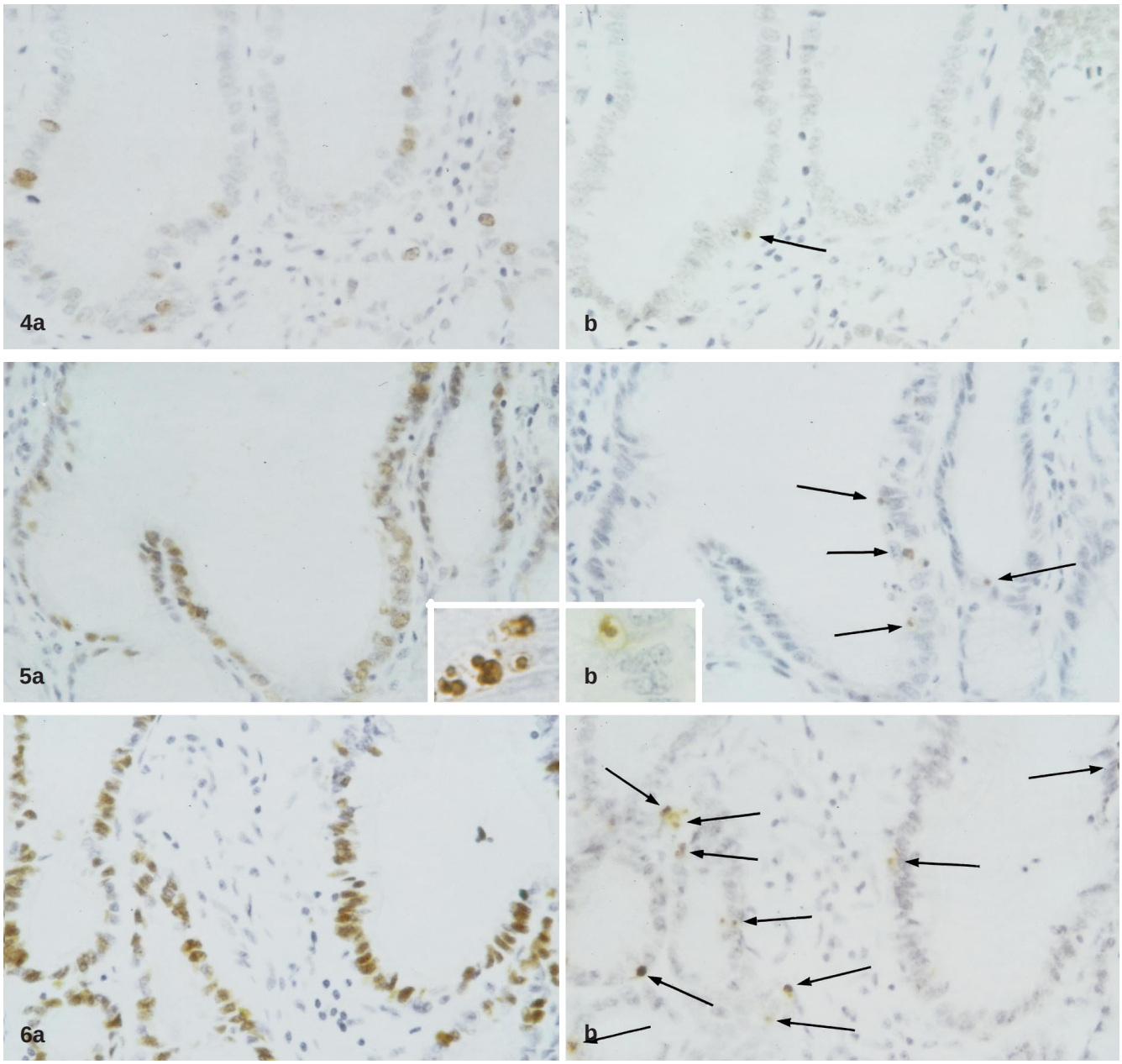
Discussion

Apoptosis occurred not only in the uppermost part of foveolar pits (luminal zone) but also (more frequently) within the G zone and in the gland zone. The apoptoses in the luminal and gland zones are related to cellular turnover; the incidence of apoptosis in the luminal zone was greater than that in the gland zone, reflecting a more rapid cellular turnover rate in the foveolar pit than in the glandular portion [8]. However apoptosis in the G-zone is supposed to play a role in the control mechanism of cell proliferation even in the steady state. Quastler and Sherman [19] already pointed out a discrepancy between the cell production in the crypts and cell loss in the intestinal villi; they supposed the overproduction of cells in the G-zone may be balanced by the cell loss within the crypt epithelium.

The apoptosis in the G-zone of the stomach was much more common in dogs than in mice (data not shown), for unknown reasons. In the human gastric mucosa, apoptosis in the G zone was recently described to be rare [9]. We recently found that cell proliferation of a human gastric cancer cell line remained almost unaffected by intermittent exposure to BrdU (unpublished data). These suggest that susceptibility of proliferative cells to apoptosis may vary remarkably from species to species and between in vivo and in vitro systems.

To study the regulation of the occurrence of apoptosis in the canine pyloric gland, we carried out flash and intermittent labelling of proliferating cells with BrdU, and detected BrdU labels in apoptoses. At light microscopic level, difficulty in unequivocal detection of BrdU labels in condensed apoptotic nuclei has hampered quantification of the labels [20]. We found that the detectability of BrdU label has much improved by using ×100 objective lens and light nuclear counterstain (Fig. 4). But it may be safe to consider our labelling indices of apoptoses as a minimum estimation. As a result, at least 30% of the apoptoses in the G-zone were flash-labelled and considered to be generated in and just after the S phase, as apoptosis is known to be recognized as soon as a few minutes after the induction. There are several reports pointing out that the apoptosis related to the G_2 checkpoint occurs preferentially in or just after the S phase [6, 12].

If the apoptosis in the G-zone occurred exclusively during the S phase, most of the apoptoses in the G-zone



Figs. 4–6 Serial sections of the G-zone of canine stomach after single injection (**Fig. 4**) and intermittent injections of BrdU for 25 h (**Fig. 5**) and 49 h (**Fig. 6**); staining incorporated **a** BrdU and **b** TUNEL labels. *Arrows* in **Figs. 4b**, **5b** and **6b** indicate TUNEL-labelled apoptoses. ×400 *Inset* in **Fig. 5a** shows clustered apoptoses labelled with BrdU. ×1000 *Inset* in **Fig. 5b** shows higher magnification of a TUNEL-labelled apoptosis. ×1000

should have been labelled by intermittent labelling for 25 h, that is longer than the reported life time of apoptosis (12–18 h [24]). The actual labelling index was 67.2%. This fact suggests that some apoptoses occur in the cell cycle phase other than the S phase, possibly relating to the G_1 checkpoint [10]. As it was estimated to take at least 28 h for labeled S-phase cells to reach the G_1 /S interphase (G_1 checkpoint) calculated from the reported cell cycle time of canine gastric gland [23], the cells traversing the G_1 checkpoint may be largely non-labelled at the time point of 25 h after the beginning of BrdU exposure, while only a small proportion of BrdU-incorporated cells may reach the G_1 checkpoint. This may be the reason why the incidence of apoptosis and mitosis in the G zone did not significantly alter by single or intermittent exposure to BrdU for up to 25 h.

After 49 h of intermittent labelling of BrdU, the incidence of apoptosis increased significantly, accompanied by cell cycle arrest (decreased mitotic index). By this time point, most of the BrdU incorporating cells reach the G_1 checkpoint and some G_1-apoptosis may occur at this checkpoint probably due to the presence of DNA mispairs. The amount of DNA mispairs may increase dramatically in cells that have passed the G_1 checkpoint

as these cells incorporate more BrdU using the BrdU-incorporated template DNA. In this circumstance, mispairs between DNA strands and DNA strand breaks may occur so frequently that many proliferative cells undergo apoptosis during the S phase or at the G_2 checkpoint.

DNA mispairs elicited by BrdU have been underestimated in studies using cell culture systems [2, 11, 17]. Our results suggest that the proliferative cells in vivo may be much more sensitive to DNA mispairs than those in cell lines, which may have partially lost the checkpoint function during the process of immortalization. It is also important to demonstrate to what extent p53 plays a role in the induction of apoptosis by BrdU. To approach this point, we are now trying to detect p53 products in our material.

The increased occurrence of apoptosis in the G zone and the probable elongation of cell cycle time induced by BrdU incorporation may result in reduction of cell production. The reduced incidence of non-labelled apoptosis after 49 h of intermittent labelling in the luminal and the gland zones suggests that the occurrence of apoptosis in these zones is down-regulated to compensate the reduced cell production.

In summary, BrdU-induced apoptosis elicited by intermittent exposure to BrdU can be used for an in vivo assessment of checkpoint function and regulation mechanism between the cell death of mature cells and cell proliferation in experimental animal systems.

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