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Original

Immunohistochemical Demonstration of S-phase Cells in Canine and Feline Spontaneous Tumors by Anti-bromodeoxyuridine Monoclonal Antibody

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Abstract: Bromodeoxyuridine (BrdU) labeling of S-phase cells, which are DNA- synthesizing cells, was demonstrated in 30 canine and 6 feline neoplasms. Simultaneously, the optimization of the BrdU-labeling method, including tissue fixation, administration dose of BrdU, and the immunohistochemical procedure, was examined. BrdU-incorporated cells in a neoplasm of a dog administered 3 mg/kg of BrdU could be detected. Although BrdU-positive cells could be visualized in 70% ethanol fixed samples, we could satisfactorily demonstrate BrdU-immunoreactivities in samples fixed in 10% buffered formalin and subjected to enzymatic treatment and acid hydrolysis. The BrdU-labeling index (LI) of malignant neoplasms tended to be higher than that of benign neoplasms. The mean BrdU LI of malignant neoplasms was significantly higher than that of benign neoplasms ($P<0.05$). The present study demonstrated that the BrdU-labeling method may be useful for detecting S-phase cells in canine and feline tissues. (J Toxicol Pathol 2005; **18**: 135–140)

Key words: Bromodeoxyuridine, S-Phase cell, dogs, cats, neoplasms

Introduction

Uncontrolled, abnormal growth is an important characteristic of neoplasia, and an analysis of the growth potential is helpful in evaluating malignancy and in estimating prognosis. Autoradiography using tritium-labeled thymidine improved the analysis of cell kinetics[1]; however, the application of radioactive thymidine in conventional laboratories is restricted. Gratzner *et al*. established monoclonal antibodies against bromodeoxyuridine (BrdU), which is an analog of thymidine, and this monoclonal antibody has been applied to non-radioactive immunohistochemical detection of S-phase cells[2]. Although the cell kinetics of selective canine neoplastic tissues have been studied using BrdU-incorporating techniques[3,4], the dose of BrdU administration, the effects of fixation and the appropriate pretreatment for antigen retrieval have not been validated in canine or feline tissues.

In this study, we applied the BrdU-labeling method to canine and feline spontaneous neoplastic tissues and optimized the administration dose and tissue fixation procedure for demonstration of BrdU-positive cells.

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Materials and Methods

Optimization of fixation

To evaluate the effects of fixation on BrdU-immunoreactivty, the small intestines of a euthanized dog and cat were sliced, 5 mm thick, and fixed in 10% buffered formalin or 70% ethanol at room temperature (RT) for 6, 12, and 24 h, 1, 2 and 3 weeks, and 1, 2 and 3 months. Moreover, samples were also fixed in 10% buffered formalin at 60°C for 2 h. One hour prior to euthanasia, these animals were administered 15 mg/kg body weight of BrdU. The euthanized dog was a mongrel and the cat was a domestic short hair, and each animal was clinically healthy. The animals were handled in accordance with the guidelines for animal experimentation of the Faculty of Applied Biological Sciences, Gifu University.

Canine and feline neoplastic tissues and administration of BrdU

The samples used were thirty canine and six feline neoplastic tissues which were surgically excised at the Veterinary Teaching Hospital of Gifu University, or at private animal hospitals. The mean age of the tumor-bearing

Table 1. Animals and Dose of BrdU Administration

Animal	No.	Sex	Age (year)	Dose of BrdU (mg/kg)	Site	Histological Diagnosis (mg/kg)
Dog	1	F	8	16.6	Mammary gland	Adenoma
	2	F	8	33.3	Mammary gland	Adenoma
	3	F	10	25.0	Mammary gland	Adenoma
	4	F	6	15.0	Mammary gland	Adenocarcinoma
	5	F	10	15.0	Mammary gland	Adenocarcinoma
	6	F	10	12.0	Mammary gland	Adenocarcinoma
	7	F	12	16.7	Mammary gland	Adenocarcinoma
	8	F	13	15.0	Mammary gland	Adenocarcinoma
	9	F	17	22.7	Mammary gland	Adenocarcinoma
	10	F	Adult	3.3	Mammary gland	Adenocarcinoma
	11	F	9	13.2	Foreleg	Squamous cell carcionoma
	12	F	12	33.3	Submandible	Squamous cell carcionoma
	13	M	Adult	15.0	Skin	Squamous cell carcionoma
	14	F	8	16.7	Neck	Sebaceous adenoma
	15	F	8	15.0	Face	Sebaceous adenoma
	16	M	11	15.0	Neck	Sebaceous adenoma
	17	F	12	33.3	Neck	Sebaceous adenoma
	18	M	Adult	33.3	Eyelid	Sebaceous adenoma
	19	M	8	33.3	Neck	Sebaceous epithelioma
	20	F	8	15.0	Hip	Sebaceous epithelioma
	21	F	8	15.0	Eyelid	Meibomian adenoma
	22	F	17	22.7	Eyelid	Meibomian carcinoma
	23	F	10	17.8	Thorax	Lipoma
	24	M	11	3.0	Hip	Lipoma
	25	M	11	6.3	Perineum	liposarcoma
	26	M	9	7.1	Oral cavity	Mastocytoma (grade III)
	27	M	14	15.0	Perineum	Mastocytoma (grade III)
	28	M	7	9.3	Foreleg	Malignant fibrous histiocytoma
	29	F	Adult	83.3	Abdomen	Malignant fibrous histiocytoma
	30	M	5	15.0	Pelvic bone	Osteosarcoma
Cat	31	F	12	16.7	Mammary gland	Adenocarcinoma
	32	F	16	55.0	Mammary gland	Adenocarcinoma
	33	F	Adult	15.0	Mammary gland	Adenocarcinoma
	34	F	13	30.0	Muzzle	Squamous cell carcinoma
	35	F	16	55.0	Face	Trichoblastoma
	36	F	11	15.0	Oral cavity	Squamous cell carcinoma

animals was 11 years (range 5–17 years old). None of the animals had received chemotherapeutic drugs before the surgical excision of tumor. The details of the samples are provided in Table 1.

The BrdU-labeling method was performed on animals whose owners provided informed consent to the aims and methods of the study[5]. BrdU (5-bromo-2'-deoxy-uridine) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). It was dissolved, 3.0–83.3 mg, in 25 μl of dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan), and 975 μl of 0.9% NaCl solution was added. The BrdU solution was sterilized using a 0.22 μm filter. One hour before tumor excision, the animals received an intravenous injection of 1 ml/kg body weight BrdU solution[5]. Consequently, BrdU solutions were administered in doses ranging from 3.0–83.3 mg/kg body weight (Table 1).

Histopathology and immunohistochemistry

Fixed samples were embedded in paraffin wax, and serial sections (4 μm) were cut and stained with hematoxylin and eosin for histopathological diagnoses. Histopathological diagnoses were based on a previous report[6].

Immunostaining of BrdU-incorporated cells was performed following the method described by Yanai *et al*.[4]. For BrdU-immunostaining, serial sections were mounted on an aminopropyltriethoxysilane (Sigma-Aldrich Co.) treated glass slide. After dewaxing and rehydrating, the serial sections were rinsed in phosphate buffered saline (PBS) and DNA was denatured using 5 N HCl at 37°C for 30 min, before being neutralized in Palitisch's boric acid-NaCl-borate buffer (pH 7.6) at 4°C for 15 min. In the case of formalin-fixed samples, the sections were subjected to protease digestion using 0.05% protease (Nagarase, Sigma-Aldrich Co.; 37°C; 1, 3, and 5 min) in PBS. After inactivation of the endogenous peroxidase by 0.3% hydrogen peroxide in methanol at RT and blocking of nonspecific antibody binding (5% normal goat serum;

Chemicon, Temecula, CA, USA), the sections were incubated with the primary anti-BrdU monoclonal antibody (IU-4, ×10,000, Cosmo Bio Co. Ltd., Tokyo, Japan) overnight at 4°C. After rinsing three times with PBS for a total period of 1 h, the sections were then incubated with biotinylated anti-mouse IgG goat polyclonal antibody (Dako, Glostrup, Dennmark) for 30 min at RT. Following this, they were rinsed three times with PBS for a total period of 30 min, and incubated with horseradish peroxidase-conjugated streptavidin (Dako) for 30 min at RT. They were

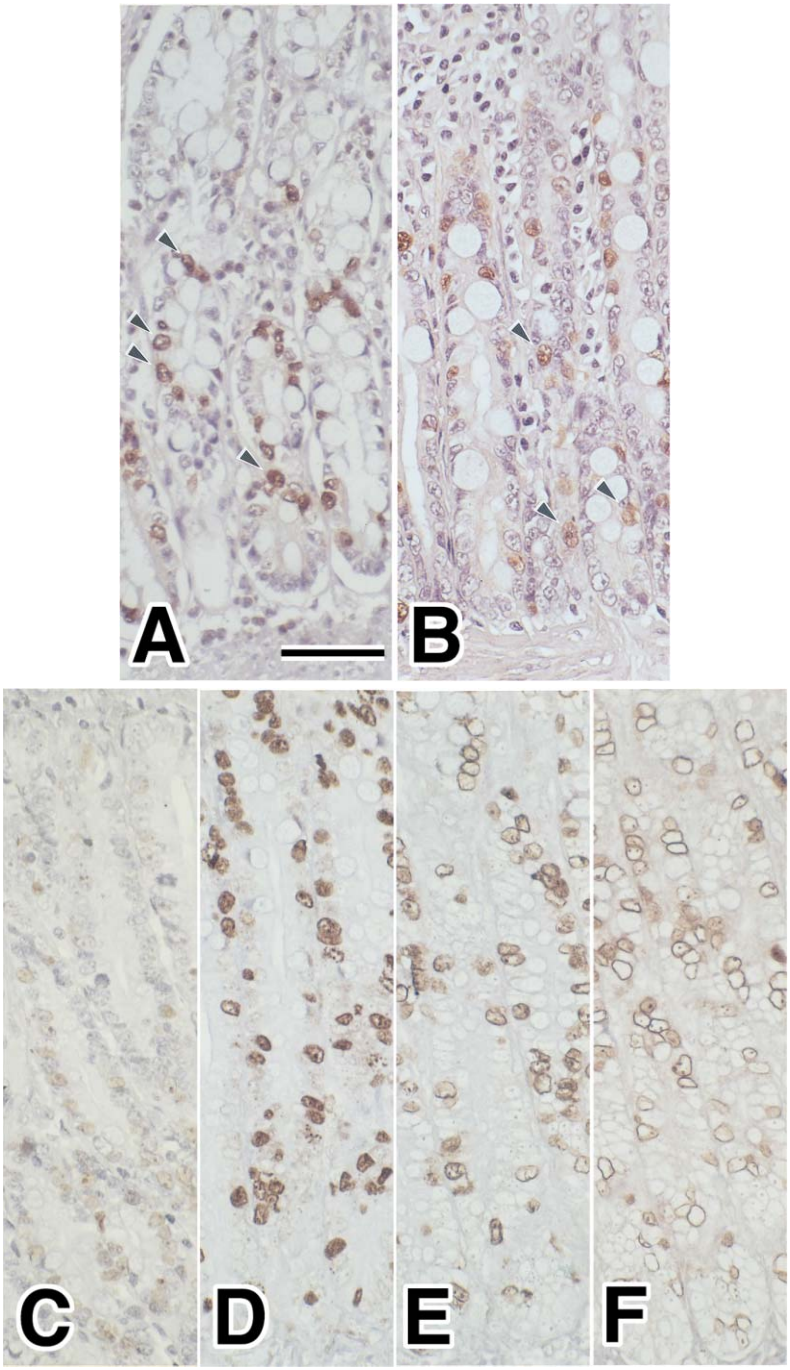

Fig. 1. BrdU-positive cells in the small intestines. **A**: feline small intestine (BrdU 15 mg/kg, i.v.) fixed with 70% ethanol for 12 h. **B**: canine small intestine fixed with 10% buffered formalin solution at 60°C for 2 h. Nuclear positive reactions were observed in epithelial cells in crypts (arrowheads). **C**: feline small intestine fixed with 10% buffered formalin solution and without protease treatment at 60°C for 2 h. Weakly positive cells were scattered in crypts. **D**: the same sample with protease treatment at 37°C for 1 min. Positive reactions were more intense than those of D, and the histological structures were preserved. **E**: the same sample with protease treatment at 37°C for 3 min. The staining intensity with hematoxylin counterstaining decreased. **F**: the same sample with protease treatment at 37°C for 5 min. Tissue damage was severe. Bar = 50 μl.

Table 2. The Effects of Fixation on BrdU-immunoreactivities

Fixation	10% buffered formalin		70% ethanol
	Protease Treatment	No Protease Treatment	
2 h, 60°C	+	+	ND
6 h, RT	+	+/–	+
12 h, RT	+	+/–	+
24 h, RT	+	+/–	+
1 w, RT	+	+/–	+
2 w, RT	+	–	+
3 w, RT	+	–	+/–
1 m, RT	+	–	–
2 m, RT	+	–	–
3 m, RT	+	–	–

+; positive, +/–; weak positive, –; negative.
h; hours, w; week (s), m; month (s), RT; room temperature, ND; not done.

Table 3. Histopathological Diagnosis and BrdU LIs

Origin	Histopathological Diagnoses	n	Means of BrdU LI (%) (ranges)
Mammary gland	Mammary carcinoma	10	17.7 (9.2–30.6)
	Mammary adenoma	3	5.6 (3.0–7.1)
Skin and appendices	Squamous cell carcinoma	5	27.3 (18.8–43.4)
	Trichoblastoma	1	4.3
	Sebaceous epithelioma	2	9.3 (8.5–10.0)
	Sebaceous adenoma	5	5.4 (2.7–7.3)
	Meibomian carcinoma	1	17.0
	Meibomian adenoma	1	3.7
Mesenchymal tissues	Mastocytoma (grade III)	2	13.5 (13.0–13.9)
	Malignant fibrous histocytoma	2	18.2 (14.5–21.7)
	Osteosarcoma	1	24.6
	Liposarcoma	1	27.5
	Lipoma	2	0.8 (0.4–1.2)
	Benign*	14	5.1 (0.4–10.0)§
	Malignant#	22	20.3 (9.2–43.4)§
Total		36	14.4 (0.4–43.4)

*; mammary adenoma, sebaceous adenoma, sebaceous epithelioma, meibomian adenoma and trichoblastoma, lipoma.
#; mammary carcinoma, meibomian carcinoma, squamous cell carcinoma, liposarcoma, mastocytoma (grade III), malignant fibrous histocytoma and osteosarcoma.
§, Significant difference between the two groups as determined by the Mann-Whitney rank test ($P<0.05$).

again rinsed three times with PBS for a total period of 30 min. The labeled cells were visualized using diaminobenzidine. Finally, the sections were counterstained using Mayer's hematoxylin. As a negative control, the primary antibody was replaced with normal mouse serum.

Assessment of immunolabeling

For comparing the intensities of BrdU-positive cells under various conditions, the intensity of the positive reactions was evaluated on the basis of three grades (+, positive; +/–, weakly positive; –, negative). Moreover, BrdU-positive cells among a total of 1000 cells (from 10 high power [× 400] fields) were counted to obtain the BrdU labeling index (BrdU LI) under the best staining condition. Significant differences between the benign and malignant neoplasms in terms of the BrdU LI were analyzed by the Mann-Whitney rank test.

Results

Optimal fixating condition

Cells incorporating BrdU were easily recognized in the tissue section. Immunoreactivity for BrdU was confined to the nucleus, and consisted of a mixture of punctuate and diffuse patterns. The staining intensity of BrdU-immunoreactivity in samples fixed with 70% ethanol for one week was the best result among all the treatment conditions (Fig. 1A). In contrast, the positive reaction observed in samples that were fixed in 10% buffered formalin for over 6 h decreased. The samples fixed in 10% buffered formalin

for 2 h at 60°C indicated a sufficient positive reaction, and the staining intensities of samples that were fixed with formalin and subjected to enzymatic digestion were equal to those of ethanol-fixed samples (Table 2). Protease digestion at 37°C for 1 min gave as the best condition. A longer digestive treatment resulted in a severe damage to the samples giving, for example, a lower intensity of counterstaining (Figs. 1 E and F). Details of the different treatment conditions are shown in Table 2.

BrdU LIs of canine and feline neoplastic tissues

Histopathological diagnoses and BrdU LIs in samples are shown in Table 3. Neoplasms were classified into mammary neoplasia (n = 13), epithelial neoplasia (n = 15), and mesenchymal neoplasia (n = 8). BrdU-labeled cells could be detected in the samples from animals administered

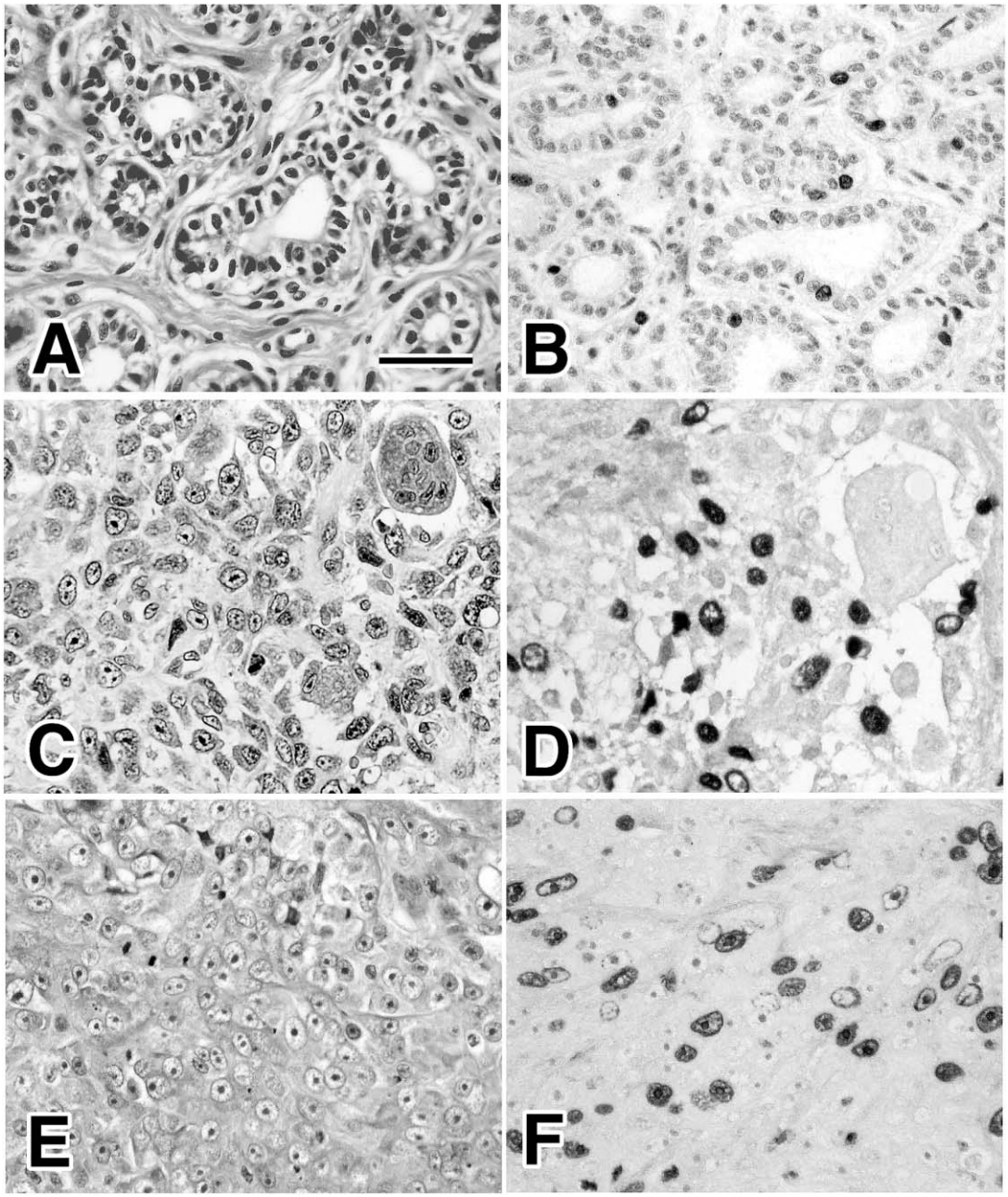

Fig. 2. BrdU-immunoreactivities in canine and feline tumors. A, C, and E: Hematoxylin and eosin staining. B, D, and F: BrdU-immunostaining. A and B: Canine mammary adenoma. Neoplastic cells formed tubular structures. Nuclei were uniform (A). BrdU-positive cells were scattered in glandular epithelial cells of tubular structures. Dosage of BrdU administration was 33.3 mg/kg. BrdU LI was 6.8%. C and D: canine osteosarcoma. Neoplastic cells appeared osteoblastic and showed severe atypia (C). Osteoblastic cells were positive for BrdU; however, osteoclastic cells were negative (D). Dosage of BrdU administration was 15.0 mg/kg. BrdU LI was 24.6%. E and F: feline mammary carcinoma. Poorly differentiated neoplastic cells had vesicular nuclei with prominent nucleoli (E). Numerous neoplastic cells were positive for BrdU (F). Dosage of BrdU administration was 15.0 mg/kg. BrdU LI was 30.5%. Bar = 50 μl.

the smallest dose of BrdU (3 mg/kg). BrdU LIs of malignant neoplasms tended to be higher than those of benign neoplasms (Fig. 2). The mean of BrdU LI of malignant neoplasms (n=22, 20.3%) was significantly higher than that of benign neoplasms (n=14, 5.1%) ($P<0.05$).

Discussion

BrdU is incorporated into nuclear DNA, as a substitite for thymidine. Raza *et al*. reported that the BrdU LI and the tritiated thymidine LI were almost the same[7]. Using enzymatic treatment and acid hydrolysis, BrdU-incorporated cells were detected even in formalin-fixed samples[8]. The best staining reactivities and good histological architectures were obtained when the tissues were fixed in 10% buffered formalin solution at 60°C for 2 h. However, in samples fixed with formalin for a longer period of time, BrdU-immunoreactivities were recovered with enzymatic treatment, although longer digestion times resulted in severe histological damages.

In experimental animals, BrdU was administered at a dose of 100–150 mg/kg body weight for immunohistochemical analysis[9,10]. However, in this study, administration of 3 mg/kg allowed the detection of BrdU-incorporated cells (Case No. 3, lipoma, BrdU LI was 0.4%). Yanai *et al*. have previously reported that BrdU-positive cells were satisfactorily demonstrated in cattle tissue that had been treated with 2 mg/kg or higher doses of BrdU and fixed in either formalin or 70% ethanol[5]. The number and staining intensity of BrdU-incorporated cells depended on the fixation or protease-digesting condition rather than the amount of administered BrdU in our study. Human patients with brain tumors were administered BrdU at doses of 500–1000 mg/day for 4–6 weeks without any serious side effects[11]. In the present study, animals that were administered BrdU also exhibited no side effects, irrespective of the dosage.

The mean BrdU LI of malignant neoplasms was significantly higher than that of benign neoplasms. BrdU LIs of malignancies tended to be higher than those of benign tumors having the same origin. Particularly, higher BrdU Ll (over 20%) was observed in squamous cell carcinoma, liposarcoma, and osteosarcoma, which have poor prognoses. Therefore, high BrdU LI may be associated with the biological behavior of malignant neoplasms.

In the present study, we demonstrated that the BrdU labeling method may be useful for detecting S-phase cells in canine and feline tissues.

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