NOTE *Clinical Pathology*

Effect of Recombinant Human Granulocyte Colony Stimulating Factor on Lymphocyte Blastogenesis in Healthy Dogs

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ABSTRACT. Effects of recombinant human granulocyte colony-stimulating factor (rhG-CSF) on the number and blastogenesis of lymphocytes were evaluated in clinically healthy dogs treated subcutaneously with rhG-CSF at a dose of 2.5 μg/kg for 3 days. Significant increases in the number of leukocytes and segmented neutrophils were observed after the administration of rhG-CSF. The number of lymphocytes also increased on days 1 and 2 after the treatment. Activities of phytohemagglutinin, concanavalin A, and pokeweed mitogen-induced lymphocyte blastogenesis (LB) were augmented to twice the pretreatment levels by the administration of rhG-CSF. These results suggested that administration of rhG-CSF activated lymphocyte functions such as LB in healthy dogs.

KEY WORDS: canine, lymphocyte blastogenesis, recombinant human granulocyte colony-stimulating factor.

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Granulocyte colony-stimulating factor (G-CSF), which is one of the humoral hematopoietic factors, stimulates not only the proliferation and differentiation of neutrophilic progenitor cells but also functions on mature neutrophils including chemotaxis, phagocytosis, and oxidative activities [6, 8, 11]. Since adverse side effects of recombinant human G-CSF (rhG-CSF) are minimized [2, 9], it is widely used as a therapeutic and prophylactic agent in both human and animal patients with certain diseases such as neutropenia, septicemia, and/or infectious diseases [2, 3, 6–9, 11, 12]. Although G-CSF is regarded as mainly acting on neutrophilic lineages [11], it has been reported that G-CSF treatment had some effects on lymphocytes in humans and animals [7, 14, 15]. G-CSF stimulated mobilization of $CD34^+$ progenitor cells and lymphocytes in humans [15], or enhanced lymphocyte blastogenesis (LB) of concanavalin A (Con A) and production of immunoglobulin in cows [14]. However, there has been few information about the influence of rhG-CSF on lymphocytes in dogs. This report deals with the effects of rhG-CSF on the number of lymphocytes and LB in healthy dogs.

Eight clinically healthy adult mongrel dogs of unknown age weighing 10 to 15 kg were purchased from the Public Health Center in Miyazaki prefecture, and acclimatized in the animal facility of the Veterinary Teaching Hospital at Miyazaki University. No abnormalities in physical conditions, complete blood count (CBC), serum biochemistry, and parasitic examinations were observed in the dogs.

RhG-CSF was obtained from Chugai Pharmaceutical Company (Tokyo, Japan). RPMI 1640, Ficoll–Isopaque (Histopaque: specific gravity; 1.077), fetal calf serum, Giemsa solution, phytohemagglutinin (PHA), Con A, and pokeweed mitogen (PWM) were purchased from Sigma (St Louis, MO, U.S.A.). CBC was taken by an automatic microcellcounter, Sysmex F-800 (Toa Medical Supply, Hyogo, Japan). The number of lymphocytes was calculated from the leukocyte differential count on a blood smear stained with Diff-Quick.

Peripheral blood mononuclear cells (PBMC) were collected by a density gradient method with Ficoll-Isopaque, washed three times with saline, and suspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum. PBMC prepared were more than 85% PBMC and 90% viability by morphological examination with Giemsa staining and trypan blue dye exclusion test, respectively.

Lymphocyte blastogenesis was evaluated by glucose consumption test as previously described [5]. In brief, each culture was composed of 100 μl of cell suspension (1×10^5 cells), 25 μl of mitogen, and 75 μl of culture medium. Both stimulated and non-stimulated (control) cultures were made in triplicate. Medium by itself was used as a negative control. Mitogens used were PHA (5 μg/ml), Con A (5 μg/ml), and PWM (10 μg/ml). The optimal concentration of each mitogen was determined in preliminary experiments. The cultures were incubated at 37°C for 96 hr, and then the glucose concentration in the supernatant from each well was measured by the method of glucose oxidation with the dry chemistry system (Fuji 5500 V, Tokyo). The stimulation index (S.I.) was calculated from the formula: S.I.=[negative control (mg/dl)–mitogen stimulated culture supernatant (mg/dl)] / [negative control (mg/dl)–control culture supernatant (mg/dl)].

After the subcutaneous injection of rhG-CSF into clinically healthy dogs at a dose of 2.5 μg/kg for 3 days (from day 0 to day 2), CBC and leukocyte differential counts were carried out at 0, 1, 2, 3, 7 and 14 days. Tests for LB were done at 0, 3, 7 and 14 days.

The statistical significance of the data between day 0 and day 14 was determined by one way repeated ANOVA measurement. A p–value less than 0.01 was considered significant.

Table 1 shows the number of circulating leukocytes and leukocyte differential counts in dogs treated with rhG-CSF. The numbers of leukocytes, segmented neutrophils, and

Table 1. The number of circulating leukocytes and differential counts in dogs treated with rhG-CSF

	Days after treatment						P
	pretreatment	1	2	3	7	14	
Leukocytes(/μl)	12400 ± 1120	32300 ± 2530	37800 ± 3120	37600 ± 3550	16500 ± 1080	14100 ± 1450	< 0.01
Band(/μl)	372 ± 75	116 ± 62	118 ± 67	55 ± 55	122 ± 60	243 ± 63	N.S.
Seg.(/μl)	6140 ± 758	22800 ± 2480	28800 ± 2760	31400 ± 2930	10600 ± 755	8760 ± 1120	< 0.01
Lym.(/μl)	3080 ± 359	5950 ± 838	5170 ± 626	2790 ± 343	3610 ± 316	3400 ± 331	< 0.01
Mono.(/μl)	1070 ± 264	1300 ± 227	1620 ± 325	1460 ± 304	1030 ± 138	944 ± 220	N.S.
Eos.(/μl)	1450 ± 267	2130 ± 400	2110 ± 454	1930 ± 397	1150 ± 175	755 ± 128	N.S.

Each number is the mean ± standard error for three experiments performed in duplicate or triplicate (n = 8). N.S. = not significant.

Table 2. Mitogen-induced lymphocyte blastogenesis in dogs treated with rhG-CSF

Mitogen	Days after treatment				P
	pretreatment	3	7	14	
PHA	1.83 ± 0.09	2.00 ± 0.13	3.66 ± 0.39	1.99 ± 0.26	< 0.01
Con A	1.27 ± 0.06	1.61 ± 0.06	2.46 ± 0.10	1.37 ± 0.07	< 0.01
PWM	1.13 ± 0.15	1.08 ± 0.04	2.37 ± 0.45	1.51 ± 0.38	< 0.01

Each number is the mean ± standard error for three experiments performed in duplicate or triplicate (n = 8).

lymphocytes were significantly increased by the rhG-CSF treatment ($p<0.01$). The number of leukocytes increased about 3-fold from the pretreatment level (day 0) after rhG-CSF administration, and then decreased to the pretreatment level by day 7. A similar kinetics of the number of segmented neutrophils was observed in dogs treated with rhG-CSF throughout the experiment period. The number of stab neutrophils tended to decrease in the rhG-CSF treated dogs. RhG-CSF administered *in vivo* stimulates proliferation and differentiation of neutrophilic lineages, thereby inducing neutrophilia which is one of the primary biological effects of G-CSF in dogs [6, 11, 12]. The number of lymphocytes also increased on days 1 and 2 after the treatment, and then returned to the pretreatment level. Many investigators reported the lymphocytotic effect of G-CSF in peripheral blood when administered *in vivo* to humans and animals [2, 4, 15, 16], though these effects were inconsistent in humans [2, 9]. RhG-CSF might not directly affect the number of circulating lymphocytes, because lymphocytes do not express G-CSF receptor [10]. In this experiment, the number of lymphocytes decreased on day 3 in spite of the supplementation of rhG-CSF, suggesting that G-CSF did not stimulate proliferation and differentiation of lymphocytic progenitor cells. Stored lymphocytes might be released from storage spaces such as bone marrow and spleen [3], from which rhG-CSF accelerated the release of mature neutrophils, since mild eosinophilia was also observed in this experiment.

Table 2 shows the PHA, Con A and PWM-induced blastogenesis of lymphocytes obtained from the dogs treated with rhG-CSF. LB activity was significantly augmented by the treatment ($p<0.01$). The S.I. values for PHA, Con A and PWM increased to double the pretreatment levels on day 7, then returned to the pretreatment levels by the 14th day of rhG-CSF administration. The enhancement of LB by rhG-CSF was consistent with the clinical cases reported previously [7]. Stabel *et al.* [14] also demonstrated the activation of lymphocyte blastogenesis, higher content of serum Ig M, and an increase in *in vitro* Ig M production of B cells in cows treated with recombinant bovine G-CSF. The mechanism of rhG-CSF in enhancing lymphocyte functions *in vivo* is not clear, because lymphocytes do not express G-CSF receptor [10]. Neutrophils have recently been shown to produce some cytokines, such as tumor necrosis factor and interleukin 1 [1]. These cytokines from activated neutrophils might stimulate cytokine network regulating lymphocyte functions, thereby inducing immunomodulation *in vivo* [1, 13, 17]. Further experiments are needed to determine the mode of action of G-CSF on the ability to immunomodulate.

G-CSF has been used for patients with neutropenia, infectious diseases, and/or malignancies [2, 3, 6–9, 11, 12]. In addition, these findings presented here suggested that administration of G-CSF *in vivo* enhances lymphocyte functions.

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