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Interleukin-8-like chemotactic factor from feline peripheral blood mononuclear cells cultured with egg white derivatives

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Abstract : The feline chemotactic factor(s) for polymorphonuclear cells (PMN) in culture supernatant from mononuclear cells (MNC) treated with egg white derivatives (EWD) were examined. Culture supernatant from MNC treated with EWD and human recombinant (hr) IL-8 remarkably enhanced chemo-taxis of feline PMN. To investigate feline chemotactic factor(s), gel electrophoresis was performed with culture supernatant from MNC treated with EWD under denaturing (18% loading gel/5% stacking gel) and nondenaturing (12.5% loading gel/5% stacking gel) condition. Hr IL-8 and culture supernatant from MNC treated with EWD yielded a distinct band in a molecular weight, 6 to 8 kDa. Eluted solution from gel slices of 6 to 8 kDa band in denaturing condition also enhanced feline PMN chemotaxis. These chemotactic activities of feline PMN induced by culture supernatant from MNC treated with EWD, hr IL-8 and eluted solution were inhibited in a dose-dependent manner by rabbit anti-feline polyclonal IgG (RAF pIgG) and monoclonal antibody (mAb) against hr IL-8. RAF pIgG also showed a binding activity with hr IL-8, suggesting that RAF pIgG against feline IL-8-like chemotactic factor(s) had cross-reactivity with human IL-8. These results suggested that feline MNC treated with EWD might release feline IL-8-like chemotactic factor(s) with a molecular weight, 6 to 8 kDa, which induces the chemotaxis of feline PMN.

Key words : cat, egg white derivatives, mononuclear cells, interleukin-8-like chemotactic factor(s).

Introduction

Many acute inflammatory responses are generally characterized first by neutrophils infiltration and later by accumulation of monocytes. The reaction is mainly mediated by inflammatory mediators produced from mononuclear cells (MNC). Of the inflammatory mediators, interleukin (IL)-8 is the one

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of the most important cytokines with chemotactic activity[1,2] to several cells such as neutrophil, lymphocytes, eosinophils, basophils, and endothelial cells[3-6]. It is a monocyte/macrophage-derived peptide that belongs to a novel cytokine family of 6 to 8 kDa[7]. The soluble products from activated monocytes and lymphocytes have been also considered to cause a cellular infiltration into inflamed sites such as arthritic joint and shown to directly induce chemotactic response for phagocytes[1,8,9]. It was shown that bacterial lipopolysaccharides (LPS), a mitogen, could modulate multiple neutrophil function and IL-1 released from monocytes was also associated with chemotaxis of phagocytes[10,11]. Although the culture supernatant of LPS-stimulated monocyte enhanced a neutrophil chemotactic activity, highly purified or recombinant IL-1 did not affect chemotactic activity of neutrophils[12]. Thus, it suggested that chemotactic activity of neutrophils was mediated by other soluble products but not by IL-1 released from MNC treated with either mitogens or immunostimulators.

Egg white derivatives (EWD) were originated from chicken egg whites[13]. All of EWD, active egg white product (AEWP) and chicken egg white-derivative immunoactive peptide (EF-203), which were supposed to stimulate macrophage- and neutrophil-functions, had also been demonstrated to enhance nonspecific immunity in mice, piglets, cattle, and rainbow trouts[14,15]. *In vitro* phagocytosis and chemotaxis of PMN were also demonstrated in the dog[13,16].

It is thought that soluble products, which are produced by MNC in response to EWD, might be associated with IL-8-like chemotactic factor(s) of PMN. Therefore, the aim of this study was to examine the feline IL-8-like chemotactic factor(s) for PMN by culture supernatant from MNC treated with EWD.

Materials and Methods

Animals : Fifteen Healthy cats of average one and half years old were housed at animal cage. All cats were kept at room temperature with 12 hours light cycle and fed on a pellet diet (Fildmaster, Purina Korea, Seoul, Korea) and tap water.

Reagents : The EWD was kindly provided by Eisai Co., Ltd. (Tokyo, Japan). EWD was filtered with a 0.45 μm-membrane before use. Hr IL-8 and mAb against hr IL-8 (IgG_1) were commercially purchased from Sigma Co. (St. Louis, MO, USA).

MNC and PMN isolation : Blood was collected in heparinized tube from jugular vein. MNC was obtained with Lymphoprep (Nycomed Pharma As, Oslo, Norway) after centrifugation at 400 × g for 40 minutes. The MNC was washed 3 times with phosphate-buffered saline (PBS). PMN was obtained from layer of erythrocyte sediment after collection of MNC. One ml of pellet of erythrocytes sediment was mixed with 10ml of 1.5% dextran (molecular weight, 200, 000 ; Wako, Osaka, Japan) in PBS. The floating cells in upper layer were collected after incubation for 60 minutes and centrifuged at 400 × g for 5 minutes. The residual erythrocytes were removed by treatment with 0.83% NH_4Cl solution for 5 minutes at 37℃ and washed 3 times with PBS. The purity of neutrophils in final PMN suspension was about 96% when determined by cytospin smear and Giemsa stain. All cells were resuspended in RPMI 1640 (Sigma Co.) supplemented with 2mM L-glutamine, 0.02mg/ml of gentamicin, and 5% fetal bovine serum (FBS ; Gibco, Grand Island, NY, USA) and finally adjusted to 2×10^6 cells/ml.

Culture supernatant : The MNC at a density of 2×10^6 cells/ml in a 24-well plate (Falcon 3047, Becton Dickinson Labware, Lincoln Park, NJ, USA) was incubated with concentration of 200 μg/ml of EWD for 24 hours at 37℃ under 5% CO_2-humidified atmosphere. The culture supernatant from MNC was collected by centrifugation (5,000 × g for 30 minutes), filtered with 0.45 μm-membrane (Millipore, Bedfold, MA, USA) and stored at -70℃ until use for assay.

Chemotaxis assay : Chemotaxis was measured by a modified Boyden chamber assay[7]. The chemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) and RPMI 1640 medium containing 1% bovine serum albumin (BSA) were prewarmed for 2 hours at 37℃ before use. Nitrocellulose filters with 120 μm thick and 3.0 μm pore size (Nihon Millipore, Yonezawa, Ibaraki, Japan) were placed on the top of the lower chamber filled with 200 μl of chemoattractant sam-

ples. Culture supernatant from MNC incubated without EWD referred thereafter to "medium alone" was added in the lower compartment as control. Then, 200 μl of PMN suspension (2×10^6 cells/ml) was placed in the upper compartment. The chambers were incubated for 45 minutes at 37℃ in 5% CO_2-humidified atmosphere. After incubation, the membrane filters were immediately taken out, stained with hematoxylin after fixation in ethyl alcohol, and mounted on glass slide. The migrated distance of PMN through the filter toward the other side was measured by microscopy at 400× magnification. Five fields for a filter were selected randomly in triplicate assay. The chemotactic responsiveness of input PMN were evaluated as absolute distance (μg/45 minutes) in the directional migration of PMN in response to chemoattractant.

Polyacrylamide gel electrophoresis : Gel electrophoresis was carried out on vertical gel of 18% polyacrylamide loading gel with 5% stacking gel in Tris-glycine buffer system, under denaturing condition. Culture supernatant from MNC treated with EWD and hr IL-8 were mixed with equal volumes of sample buffer (Laemmli sample buffer containing 5% β-mercaptoethanol; Bio-rad, Richmond, CA, USA), boiled, and applied to the gel[17]. Nondenaturing condition was done using 12.5% loading gel with 5% stacking gel. After electrophoresis at 80 volt for 3 hours, the gel was stained with a 0.025% Coomassie brilliant blue R-25018.

Electroelution : Proteins were eluted using electro-eluter (Model 422 Electro-Eluter, Bio-rad) according to the manufacturer's instructions. After electrophoresis, gel containing proteins was cut out and put into the electro-eluter and eluted at 8 to 10 mA/glass tube in the constant current for 4 hours in Tris-glycine buffer system.

Antisera : Hyperimmune antisera against feline chemotactic factor(s) were prepared in rabbits. Eluted solution containing chemotactic factor(s) was subcutaneously injected into back skin of four female rabbits (NewZealand White Rabbit; average body weight: 2.8 kg) in Freund's complete adjuvant (Sigma Co.). Booster injections with eluted solution in Freund's incomplete adjuvant (Sigma Co.) were carried out twice in 3 weeks interval. As a control, preimmune sera from these rabbits were obtained before immunization. Fourteen days after the last immunization, antisera were collected by bleeding. Antibody titers against chemotactic factor(s) were determined by ELISA.

Neutralization tests with anti-human IL-8 monoclonal antibody and rabbit anti-feline polyclonal IgG : To examine whether mAb against hr IL-8 and RAF pIgG neutralize the chemotactic activity of PMN, each of mAb against hr IL-8 and RAF pIgG diluted with various concentrations was added to the culture supernatant from feline MNC treated with EWD, hr IL-8 and eluted solution, respectively. The mixed samples were placed for 30 minutes at room temperature and the chemotactic activity of feline PMN for the mixed samples was also evaluated.

ELISA : The 96 well plates were coated with either culture supernatant from EWD-treated MNC or eluted solution or hr IL-8 in 0.1 M sodium bicarbonate buffer (pH 9.6). After washing 3 times with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with 1% gelatin in PBS at room temperature for 1 hour. RAF pIgG (0.012, 0.05, 0.19 and 0.75 mg/ml) in PBS-T was added to each well of plate. After incubation for 1 hour, 100 μl of a anti-rabbit IgG alkaline phosphate conjugate (1/4,000) were added to the washed wells and incubated for 1 hour at room temperature. Plates were washed 3 times with PBS-T and filled with 100 μl/well of alkaline phosphate substrate (p-nitrophenyl phosphate, 1.0 mg/ml in 10% diethanolamine buffer, containing 0.5 mM $MgCl_2$, pH 9.8; Sigma Co.). The enzymatic reaction was stopped by adding 0.1 M EDTA in PBS. The absorbance was measured at 405 nm in a microplate ELISA reader (Model 550, Bio-rad).

Data analysis : The statistical significance was determined by Student's *t*-test. All data expressed mean±standard error (M±SE).

Results

Gel electrophoresis : As shown in Fig 1, both hr IL-8 (lane I) and culture supernatant from MNC treated with EWD (lane II) yielded a distinct band with a molecular weight of 6 to 8 kDa under both denaturing (18% loading gel/5% stacking gel; panel A) and nondenaturing (12.5%

loading gel/5% stacking gel ; panel B) condition.

Feline PMN chemotaxis : Culture supernatant from MNC (2×10^6 cells/ml) treated with EWD (200μg/ml) for 24 hours (Fig 2) showed a significant enhancement of PMN chemotaxis as compared with control ($p<0.01$). As shown in Fig 3, hr IL-8 also enhanced the chemotactic activity of feline PMN

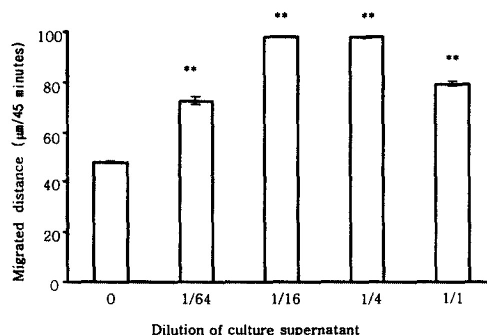

Fig 2. Chemotactic response of PMN to culture supernatant from MNC treated with EWD. The values represent M±SE, n = 3, **p ⟨ 0.01.

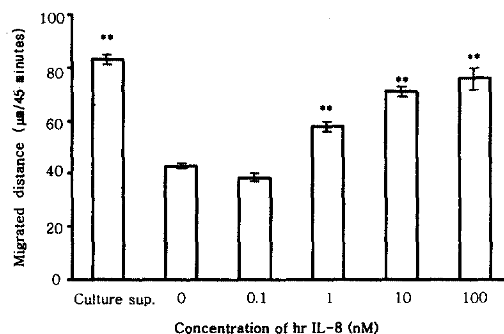

Fig 3. Chemotactic response of feline PMN to hr IL-8 and culture supernatant (1/16) from MNC treated with EWD. The values represent M±SE, n = 3, **p ⟨ 0.01.

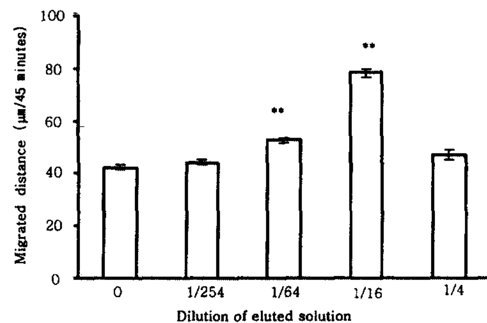

Fig 4. Chemotactic response of feline PMN to eluted solution from polyacrylamide gel after electrophoresis. The values represent M±SE, n = 3, **p ⟨ 0.01.

Fig 1. Polyacrylamide gel electrophoresis of hr IL-8 and culture supernatant from MNC treated with EWD. Panel A : Denaturing gel electrophoresis, Panel B : Nondenaturing gel electrophoresis. I : Hr IL-8, II : Culture supernatant from MNC treated with EWD. III : Size marker.

at concentrations of 0.1 to 100 nM ($p<0.01$) in a dose-dependent manner. Eluted solution (Fig 4) also enhanced the PMN chemotaxis as compared with control ($p<0.01$).

Neutralization effect of RAF pIgG and anti-human IL-8 mAb on chemotactic activity : In the neutralization effect of the chemotaxis with RAF pIgG and mAb against human IL-8, the activity with culture supernatant from MNC treated with EWD was inhibited ($p<0.05$ to 0.01) in a dose-dependent manner by addition of RAF pIgG at concentrations of 0.38 to 1.5 mg/ml and mAb against human IL-8 at concentrations of 0.5 to 5 μg/ml (Fig 5). Similarly, the inhibitory effect of RAF pIgG and mAb against human IL-8 was also observed in the chemotactic activity of feline PMN to hr IL-8 (Fig 6) and eluted solution (Fig 7). However, in the examination of the possibility of nonspecific inhibition for immunoglobulin isotype (IgG) of RAF pIgG, any chemotactic activity tested was not inhibited by addition of high concentration of 1.5 mg/ml of control pIgG instead of RAF pIgG (Fig 5 to 7).

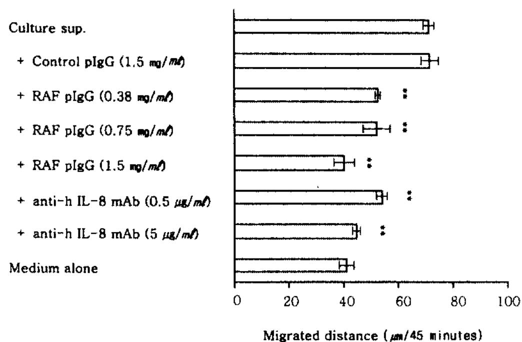

Fig 5. Effect of RAF pIgG and mAb against human IL-8 on feline PMN chemotaxis by culture supernatant (1/16) from MNC treated with EWD. The values represent M±SE, n = 3, **p < 0.01, compared to culture supernatant (1/16) from MNC treated with EWD.

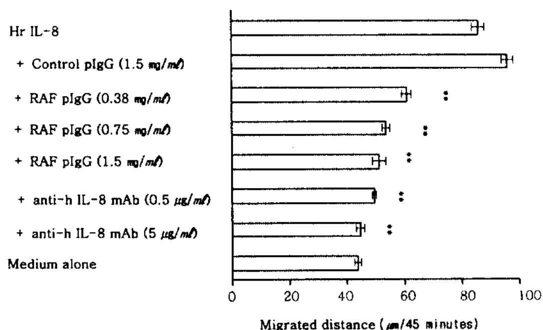

Fig 6. Effect of RAF pIgG and mAb against human IL-8 on feline PMN chemotaxis by hr IL-8 (10 nM). The values represents M±SE, n = 3, **p < 0.01, compared to hr IL-8 (10 nM).

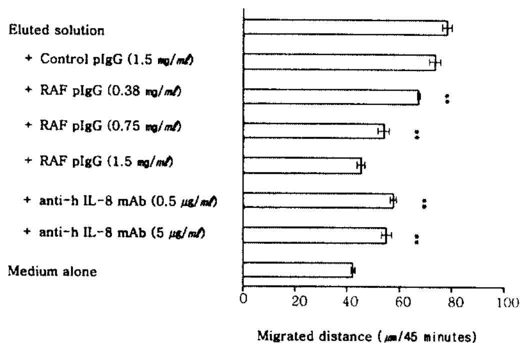

Fig 7. Effect of RAF pIgG and mAb against human IL-8 on feline PMN chemotaxis by eluted solution (1/16). The values represents M±SE, n = 3, **p < 0.01, compared to eluted solution (1/16).

Cross-reactivity of RAF pIgG to human IL-8 : The cross-reactivity of RAF pIgG with hr IL-8 was tested by ELISA. As shown in Fig 8, RAF pIgG reacted with hr IL-8 in a dose-dependent manner ($p<0.01$). Similarly, RAF pIgG also reacted with culture supernatant from MNC treated with EWD (Fig 9) and eluted solution (Fig 10), respectively.

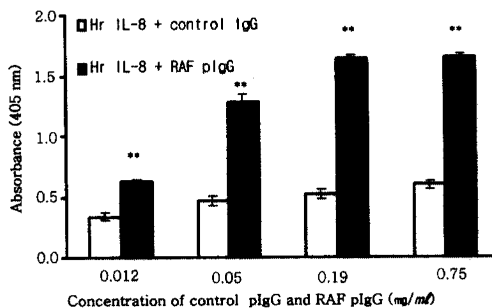

Fig 8. Absorbance at 405 nm of hr IL-8 reacted with control IgG or RAF pIgG in various concentration after alkaline phosphate reaction. The values represent M±SE, n = 3, **p < 0.01, compared to hr IL-8+control pIgG.

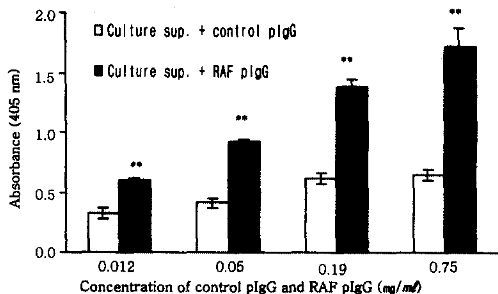

Fig 9. Absorbance at 405nm of culture supernatant (1/16) from MNC treated with EWD reacted with control IgG or RAF pIgG in various concentration after alkaline phosphate reaction. The values represent M±SE, n = 3, **$p < 0.01$, compared to culture supernatant from MNC treated with EWD+control pIgG.

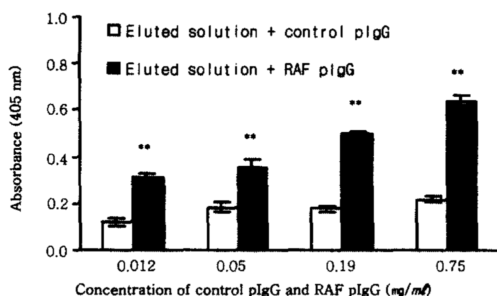

Fig 10. Absorbance at 405nm of eluted solution reacted with control IgG or RAF pIgG in various concentration after alkaline phosphate reaction. The values represent M±SE, n = 3, **$p < 0.01$, compared to eluted solution+control pIgG.

Discussion

The present results showed that culture supernatant from MNC treated with EWD highly enhanced chemotactic activity for PMN. It was also reported with culture supernatants from canine MNC treated with EWD in previous study[16]. It suggested that EWD was capable of releasing the chemoattractants from MNC consisting of monocytes and lymphocytes. It also suggested that enhancing effect of EWD on chemotactic response of feline peripheral blood PMN was mediated by humoral factor(s) produced by EWD-activated MNC.

Some cytokines have cross-reactivity between species, e.g. human IL-2[19], human IL-6[20]. Thus, it was cautiously determined that human IL-8 and mAb against human IL-8 might exert any effects on the feline PMN chemotaxis. Data from this study indicated that feline PMN chemotaxis was induced by human IL-8. In addition, mAb against human IL-8 inhibited the feline PMN chemotaxis, which was enhanced by both culture supernatant from MNC treated with EWD and hr IL-8. Thus, our results suggested that feline IL-8-like chemotactic factor(s) was existed in culture supernatant from MNC treated with EWD. PMN chemotactic responses to hr IL-8 were examined in various animal species[21]. Neutrophils of human, monkeys, hamsters, and dogs to hr IL-8 exhibited the increased chemotactic activity, but those of rabbit, rat, and mice showed a low responsiveness to hr IL-8[21-23].

Therefore, isolation and characterization of feline IL-8-like chemotactic factor(s) were needed to define the mechanisms of PMN chemotaxis by the culture supernatant from MNC treated with EWD. Hr IL-8 and culture supernatant from MNC treated with EWD were examined in SDS-PAGE under denaturing and nondenaturing conditions. All of two conditions showed a distinct band of 6 to 8 kDa. The human IL-8 has been reported as a 6 to 8 kDa[24]. It was demonstrated that chemotactic activities of feline PMN induced by all of culture supernatant from MNC treated with EWD, hr IL-8, and eluted solution were inhibited by RAF pIgG and mAb against hr IL-8. Therefore, these results suggested that feline IL-8-like chemotactic factor(s), 6 to 8 kDa proteins, were recognized by both RAF pIgG and mAb against human IL-8 and were in part agreement with the reports that chemotactic activities by culture supernatant from bovine MNC treated with LPS were inhibited by ascites containing anti-human IL-8 antibody[25]. RAF pIgG showed a high reactivity with hr IL-8, suggesting that RAF pIgG has cross-reactivity to human IL-8. In another study[26], however, polyclonal antibodies against canine IL-8 did not inhibit the enhanced chemotactic responses of canine PMN by hr IL-8.

In summary, present study suggested that EWD could stimulate nonspecific immune response in cat by releasing IL-8-like chemotactic factor(s) with a molecular weight 6 to 8 kDa. These chemotactic factor(s) were produced from MNC treated with EWD and induced PMN chemotaxis. The elu-

cidation of feline IL-8-like chemotactic factor(s) produced from feline MNC exposed to EWD will be considerably important in the study of animal cytokines as well as immunostimulators.

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계난백유래물질로 배양한 고양이 말초혈액 단핵구세포에서 분비되는 interleukin-8 樣 유주성인자

이 재 권 · 양 만 표

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(2000년 5월 3일 게재승인)

국문초록 : 계난백유래물질(EWD)로 배양한 고양이 말초혈액 단핵구세포(MNC)의 배양상층액에서 다형핵백혈구(PMN)에 대한 유주성인자를 조사하였다. EWD로 배양한 MNC 배양상층액과 human recombinant (hr) interleukin (IL-8)는 고양이 PMN의 유주성을 현저하게 증가시켰다. 이 유주활성물질을 규명하기 위해 EWD 처리 MNC 배양상층액을 gel electrophoresis(denaturing 조건 18% loading gel 및 nondenaturing 조건 12.5% loading gel)를 실시한 결과, EWD로 배양한 MNC 배양상층액과 hr IL-8는 모두 분자량 6~8kDa에서 band를 나타내었다. Denaturing 조건에서 6~8kDa band의 gel slices에서 용출시킨 용출액에서도 고양이 PMN에 대한 유주활성이 인정되었다. EWD로 배양한 MNC 배양상층액, hr IL-8 및 용출액에 의해 유도된 고양이 PMN의 유주활성은 rabbit anti-feline polyclonal IgG(RAF pIgG)와 hr IL-8에 대한 mAb에 의해 농도의존적으로 억제되었다. 또한 RAF pIgG는 hr IL-8와 결합을 보임으로써 사람의 IL-8와 교차반응을 시사하였다. 이상의 결과로부터 EWD로 배양한 고양이 MNC는 분자량 6~8kDa의 IL-8 樣 유주성인자를 분비하여 PMN의 유주성을 유도하는 것으로 사료되었다.

Key words : cat, egg white derivatives, mononuclear cells, interleukin-8-like chemotactic factor(s).