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Effect of 1,2-Benzopyrone on Chemotactic Activity of Peripheral Blood Leukocytes in the Dog

Jee-hyun Kim, Ji-houn Kang and Mhan-pyo Yang[1]

Laboratory of Veterinary Internal Medicine, College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea

Abstract: This study was undertaken to examine whether 1,2-benzopyrone affects on chemotactic activity of canine peripheral blood leukocytes. A modified Boyden chamber method was used on chemotaxis evaluation. The direct treatments of 1,2-benzopyrone showed no effects on the chemotaxis of peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs). But chemotaxis of PMN was remarkably enhanced by culture supernatant from PBMC but not PMN treated with 1,2-benzopyrone. Similarly, it was also increased by recombinant (r) interleukin (IL)-8. This chemotactic activity of PMN was inhibited by addition of anti-rIL-8 polyclonal antibody. The chemotaxis of PBMC was not enhanced by culture supernatant from either PBMC or PMN treated with 1,2-benzopyrone. Therefore, these results suggested that the chemotactic activity of PMN may be mainly mediated by IL-8-like factor(s) produced from PBMC treated with 1,2-benzopyrone.

Key words : dog, 1,2-benzopyrone, chemotactic activity, leukocytes.

Introduction

Polymorphonuclear cells (PMNs) are the main effector cells which are involved in the immune response to microorganisms. PMNs move to the site of infection or inflammation by factors generated by the interaction of host cells and infecting pathogens. They are attracted by soluble factors termed chemoattractants[14]. Chemoattractants cause them to migrate and adhere to the endothelium via cell-surface receptors[3]. Directed migration of leukocytes along chemical gradient is fundamental to development of lymphatic tissues, lymphocyte recirculation and accumulation of leukocytes at sites of inflammation or tissue injury[24]. 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[1,4,19]. The cytokines such as IL-1 and -8 are also considered to be the predominant neutrophil chemoattractant, which actively participates in the induction of the inflammatory diseases[2].

1,2-benzopyrone (C9H6O; Coumarin) is a naturally occurring fragrance compound found in various plants and essential oil such as tonka beans and lavender oil[9]. It was known that 1,2-benzopyrone activates directly macrophages[6]. 1.2-benzopyrone has been used for treatment of high protein lymphedema[12]. Since 1,2-benzopyrone highly binds to plasma proteins, proteins binding with 1,2-benzopyrone are likely to be transported through microvessels into the tissue spaces[8]. Protein complex is subsequently phagocytosed by macrophages and thereby reducing extravascular protein and decreasing colloid pressure in the tissue spaces[17]. It has been also studied clinically as therapeutic agents related to malignant melanoma, renal cell carcinoma, and specific inflammatory disease[7].

It was suggested that 1,2-benzopyrone has an immunoenhancing effect on the phagocytic activity and oxidative burst activity (OBA) of PMN. The phagocytic activities of PMN as well as monocyte-rich cells were enhanced by addition of culture supernatant from peripheral blood mononuclear cells (PBMCs) treated with 1,2-benzopyrone[26]. When latex beads are added to PMN, the OBA of latex beads-phagocytized PMN was remarkably enhanced by culture supernatant from PBMC but not PMN treated with 1,2-benzopyrone[20]. It was therefore assumed that the PBMC stimulated by 1,2-benzopyrone may release many factors associated with immune response and host defense. Thus, the aim of present study is to examine the immunostimulating effects of 1,2-benzopyrone on chemotaxis of canine peripheral blood leukocytes.

Materials and methods

Animals

Male, clinically healthy three Beagle dogs born from the same mother, ages of approximately 2 years, were used as blood donors. All dogs were housed in room temperature (22±2), maintaining day (12 h) and night (12 h) rhythm. All dogs were individually managed in cages and fed a pellet diet (Merry dog, Purina Korea, Seoul, Korea).

Reagents

1,2-benzopyrone (Sigma-Aldrich Co., St. Louis, MO, USA) was diluted with dimethyl sulfoxide (DMSO; Sigma-Aldrich Co.) and passed through a 0.45 μm-membrane filter (Millipore Co., Bedford, MA, USA) before use as stock solution. Porcine recombinant (pr) IL-8, goat anti-pr IL-8 polyclonal antibodies (pAb) (IgG) (R&D systems Inc., Minneapolis, MN, USA) and

[1]Corresponding author.
E-mail : mpyang@chungbuk.ac.kr

rabbit anti-HRV G4 (ST-3) pAb (IgG) were commercially purchased.

PBMC and PMN isolation

Peripheral blood drawn in heparinized tube from jugular vein was layered on the equal volume of Percoll solution (specific gravity, 1.077; Percoll™, Amersham Bio., AB, Sweden) and centrifuged at 400×g for 40 min at room temperature. The resulting PBMC in the interface between plasma and Percoll solution layer was harvested, and treated with 0.83% NH_4Cl Tris-base buffer (pH 7.2) for 5 min to lyse remaining erythrocytes. The PBMC was composed of both approximately 30% monocytes and 70% lymphocytes when determined in cell counting by Wright-Giemsa staining. The PMN was obtained from layer of erythrocyte sediment after collection of PBMC. One milliliter of the upper part of the erythrocytes was mixed with 10 ml of 1.5% dextran (molecular weight, 200,000; Wako Ltd, Japan) in PBS and allowed to sediment for 45 min. The residual erythrocytes were lysed by treatment with 0.83% NH_4Cl solution for 5 min at 37°C and washed 3 times with PBS. The purity of neutrophils in final PMN suspension exceeded 96% when determined by cytospin smear and Diff-Quick stain. The viability of PBMC and PMN determined by a trypan blue dye exclusion test was always more than 97%. All cells were resuspended in RPMI 1640 medium (Gibco Co., USA) supplemented with 2 mM L-glutamine, 0.02 mg/ml of gentamicin and 5% fetal bovine serum (Gibco Co.) and finally adjusted to 2×10^6 cells/ml.

Culture supernatant

The isolated PMN and PBMC at density of 2×10^6 cells/ml in a well of a 24-multiwell plate (Nucn Co. Naperville, IL, USA.) were incubated with a concentration of 100 μM of 1,2-benzopyrone (Sigma-Aldrich Co.) with minimal volume (<10%) of dimethyl sulfoxide (DMSO; Sigma-Aldrich Co.) for 24 h at 37°C under 5% CO-humidified atmosphere. The supernatant was collected by centrifugation at 5,000×g for 10 min, filtered with 0.45 μm-pore size membrane filter and stored at -70°C until use for assay.

Chemotaxis assay

Chemotactic activities for PMN and PBMC were determined as migration distance in Millipore membrane filters by a modified Boyden chamber method[30]. The chemotaxis chamber (Neuro probe, MD, USA) and RPMI 1640 medium containing 1% bovine serum albumin (BSA; Sigma-Aldrich Co.) were prewarmed for 2 h at 37°C. Lower chamber was filled with 200 μl of 1,2-benzopyrone at various concentrations, culture supernatant from PMN or PBMC treated with 1,2-benzopyrone, and rIL-8, respectively. Control was filled with culture supernatant treated without 1,2-benzopyrone. A nitrocellulose filter (Nihon Millipore, Yonezawa, Ibaraki, Japan) with 120-μm thickness and 3.0-μm pore size was placed on the top of well of the lower compartment. And then, 200 μl of PMN or PBMC (2×10^6 cells/ml) suspension was put into the upper compartment. The chambers were incubated for 45 min at 37°C in 5% CO_2-humidified atmosphere. After incubation, nitrocellulose filters were immediately taken out, fixed in ethyl alcohol, stained with hematoxylin, and mounted on slide glass. The migrated distance of cells through membrane 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 responses of input cells were evaluated as absolute distance (μm/45 min) in the directional migration of cells to chemoattractant.

Data analyses

The Student's *t* test was used for statistical significance determinations. All data were expressed as mean±standard error of mean (S.E.M.).

Results

Direct effect of 1,2-benzopyrone on chemotaxis of peripheral blood leukocytes

To examine the direct effect of 1,2-benzopyrone on chemotactic activity of canine peripheral blood leukocytes, the migrated distance of PMN and PBMC for 1,2-benzopyrone was measured. The direct treatments of 1.2-benzopyrone at concentration of 1 to 500 μM showed no effects on chemotaxis of PMN (Fig 1A) and PBMC (Fig 1B) as compared with

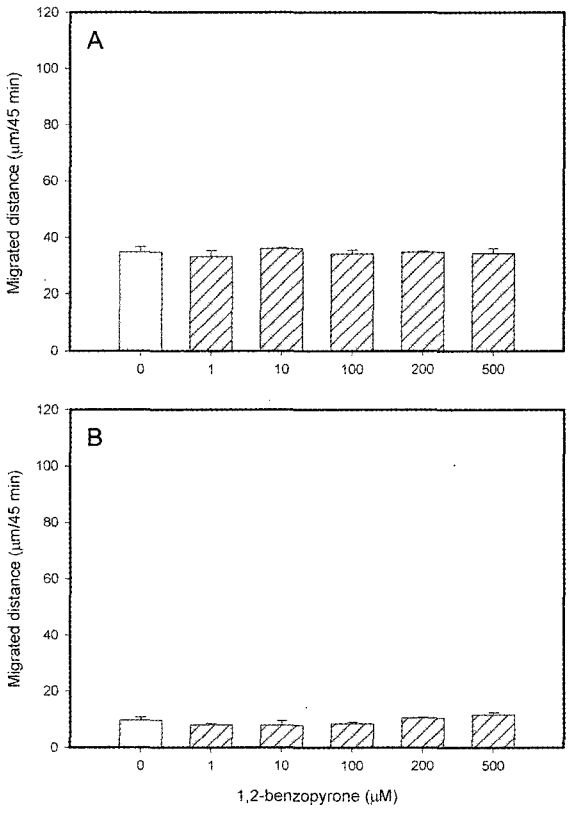

Fig 1. The direct effect of 1,2-benzopyrone on PMN (A) and PBMC (B) chemotaxis. The data represent mean±S.E.M. (n=3).

controls treated without 1.2-benzopyrone, respectively.

Effect of culture supernatant from PMN or PBMC treated with 1,2-benzopyrone on chemotaxis of peripheral blood leukocytes

To examine the stimulating effect of culture supernatant from PMN or PBMC treated with 1,2-benzopyrone on chemotaxis of peripheral blood leukocytes, the migrated distance of PMN or PBMC for each culture supernatant was measured. PMN (Fig 2A) and PBMC (Fig 2B) did not show any migration in response to the culture supernatant from PMN treated with 1.2-benzopyrone when compared to control, respectively. Overall, PMN showed a tendency to migrate more than PBMC. However, culture supernatant from PBMC treated with 1,2-benzopyrone enhanced remarkably the chemotaxis of PMN ($P<0.01$) when compared to that of control. This chemotactic activity was peaked at 25% of culture supernatant from PBMC (2×10^6 cells/ml) treated with 1,2-benzopyrone (100 μM) for 24 h in a dose-response curve (Fig 3A). But the PBMC in response to this culture supernatant showed very low activity (Fig 3B).

Chemotactic response of PMN by IL-8

The chemotactic activity of PMN to rIL-8 was also examined. As shown in Fig 4, rIL-8 enhanced the chemotactic activity of PMN at concentrations of 1 to 20 nM ($p<0.01$) when compared to control. This activity of PMN to rIL-8 at 10 nM was equivalent to that of culture supernatant (25%) from PBMC treated with 1,2-benzopyrone.

Neutralization test with anti-IL-8 pAb

To examine whether the enhanced chemotactic activity of PMN to culture supernatant from PBMC treated with 1,2-benzopyrone is due to IL-8, the neutralization test using the

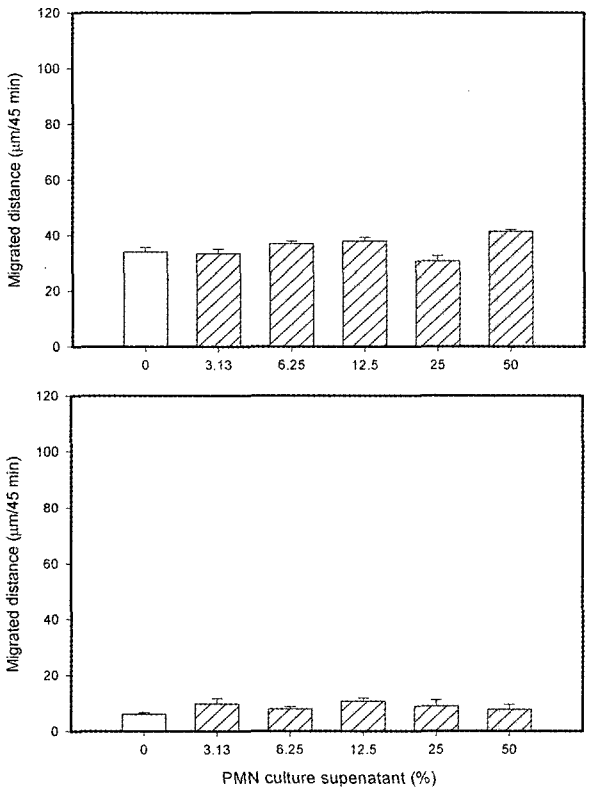

Fig 2. Chemotactic response for PMN (A) and PBMC (B) by culture supernatant from PMN (2×10^6 cells/ml) treated with 1,2-benzopyrone (100 μM) for 24 h. The data represent mean± S.E.M. (n=3).

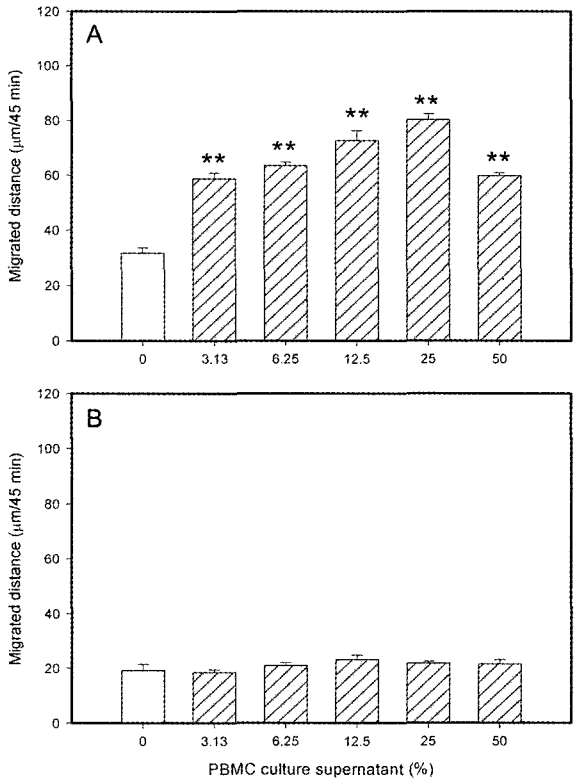

Fig 3. Chemotactic response for PMN (A) and PBMC (B) by culture supernatant from PBMC (2×10^6 cells/ml) treated with 1,2-benzopyrone (100 μM) for 24 h. The data represent mean± S.E.M. (n=3). **p<0.01, compared to control.

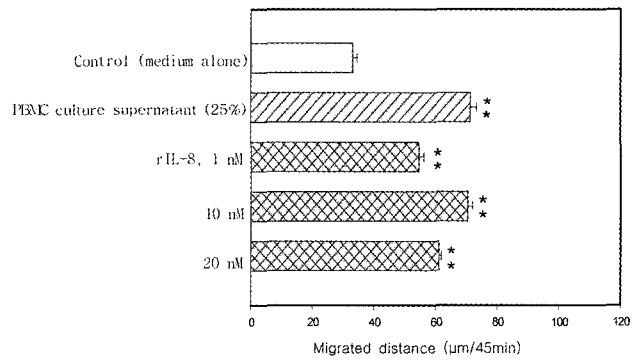

Fig 4. Chemotactic response of PMN to rIL-8. The data represent mean±S.E.M. (n=3). **p<0.01, compared to control (medium alone).

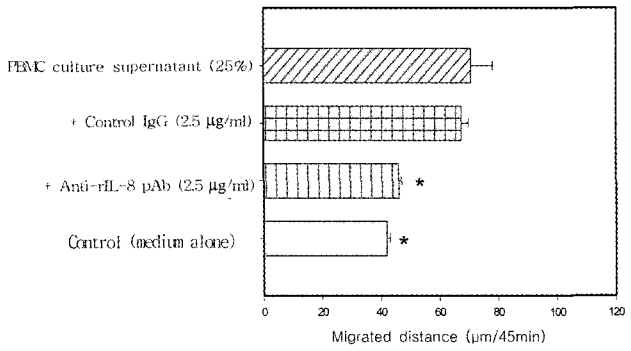

Fig 5. Effect of anti-rIL-8 pAb on PMN to culture supernatant (25%) from PBMC treated with 1,2-benzopyrone. The data represent mean±S.E.M. (n=3). *$p<0.05$, compared to culture supernatant (25%) from PBMC treated with 1,2-benzopyrone.

anti-rIL-8 pAb was performed. As shown in Fig 5, the chemotactic activity of PMN to culture supernatant from PBMC treated with 1,2-benzopyrone was inhibited ($p<0.05$) by addition of anti-rIL-8 pAb at concentration of 2.5 μg/ml when compared with that of positive control (culture supernatant (25%) from treated with 1,2-benzopyrone). However, in the examination of the possibility of nonspecific inhibition for immunoglobulin isotype, IgG, of anti-rIL-8 pAb, any chemotactic activity of PMN to culture supernatant from PBMC treated with 1,2-benzopyrone was not inhibited by addition of concentration of 2.5 μg/ml of control IgG.

Discussion

In previous study[26] associated with phagocytic activity of canine peripheral blood leukocytes, PBMC and PMN at high concentrations of 1.2-benzopyrone such as 200 to 500 μM showed the reduced viability of leukocytes. Therefore, 1,2-benzopyrone in this study was used at concentration of 100 μM showing high cell viability and no cytotoxic effect.

1,2-benzopyrone has directly no effects on the migration of freshly prepared PMN. It is conceivable that antigenic components of 1,2-benzopyrone may be less responsible for PMN chemotaxis. Culture supernatant from PMN treated with 1,2-benzopyrone was also not active for PMN. This finding was consistent with the facts that PMN does not release chemotactic or phagocytic factors by any antigenic or mitogenic stimulation[15,16,20,26].

The present results showed that the culture supernatant from PBMC treated with 1,2-benzopyrone highly enhanced the chemotactic activity for PMN. It may be supported that 1,2-benzopyrone is capable of releasing the chemoattractants from PBMC consisting of monocytes and lymphocytes. It is, therefore, suggested that the enhancing effect of 1,2-benzopyrone on chemotactic activity of PMN was mediated by soluble product(s) released from 1,2-benzopyrone-stimulated PBMC. Certain cytokines are capable of crossing the species barrier. Thus, it was cautiously determined that porcine IL-8 and pAb against IL-8 might exert any effects on the canine PMN chemotaxis. The chemotactic activity for canine PMN by porcine IL-8 was equivalent to that of culture supernatant from PBMC treated with 1,2-benzopyrone. In addition, polyclonal antibody against porcine IL-8 inhibited the chemotactic activity of canine PMN, which was enhanced by culture supernatant from PBMC treated with 1,2-benzopyrone. These results suggested that the chemotactic factor(s) were existed in soluble products from PBMC treated with 1,2-benzopyrone. These soluble products from PBMC treated with 1,2-benzopyrone also stimulated phagocytosis and oxidative burst activity of canine peripheral blood phagocytes[20,26].

The representative chemokines produced from activated PBMC are known as IL-8 and IL-1. However, although the culture supernatant of LPS-stimulated monocytes enhanced PMN chemotactic activity, highly purified or recombinant IL-1 did not affect chemotactic activity of PMN[23]. It was reported that the chemotactic activity for PMN in culture supernatant from PBMC exposed to egg derivatives (EWD) or conjugated linoleic acid (CLA), immunostimulators, is identified as IL-8 with molecular weight 6 to 8 kDa[21,31,32]. Therefore, it could be thought that soluble products, which was produced from PBMC in response 1,2-benzopyrone, will be associated with canine IL-8-like chemotactic factor(s) of PMN.

On the other hand, culture supernatant from PBMC treated with 1,2-benzopyrone was not active for PBMC. This may be, in part, associated with PBMC populations. Because the populations of PBMC isolated from canine peripheral blood were composed of both approximately 30% monocytes and 70% lymphocytes. The chemotactic responsiveness of monocytes in response to chemoattractants is evaluated for 90 to 120 minutes in the directional migration of Boyden chamber assay[5,25]. Therefore, another possibility is that the migration rate of PBMC containing monocytes is much slower than that of PMN.

IL-8 has multiple functions. This peptide stimulates neutrophil chemotaxis and modulates neutrophil and endothelial interaction[10,22]. It induces lysosomal enzyme release from neutrophils, expression of adhesion molecules on neutrophils[11,29]. The genome sequence of canine IL-8 shares 75, 80, or 85% identity with the human, rabbit, or porcine IL-8, respectively[18]. Therefore, isolation and characterization of canine IL-8-like factor(s) were needed to define the mechanisms of PMN chemotaxis by soluble products from PBMC treated with 1,2-benzopyrone. IL-8 appears to play an important role in pathogenesis of acute and chronic inflammatory diseases such as rheumatoid arthritis, cystic fibrosis, and systemic fungal infection[13,27]. Treatment with anti-IL-8 antibody reduced chemotactic activity by synovial fluids of rheumatoid arthritis patients[28]. For the clinical application, the co-administration of 1,2-benzopyrone will be able to augment the host defense in animals with diseases including lymphedema and immunodeficiency or no response to antibiotic treatment. This study suggested that the mostly enhancing effect of 1,2-benzopyrone on chemotactic response of canine PMN is mediated by IL-8-like

humoral factor(s) produced from PBMC treated with 1,2-benozpyrone.

Conclusion

The direct treatments of 1,2-benzopyrone showed no effects on the chemotaxis of PBMC and PMN. The chemotaxis of PBMC was not enhanced by culture supernatant from either PBMC or PMN treated with 1,2-benzopyrone. The chemotaxis of PMN was enhanced by culture supernatant from PBMC but not PMN treated with 1,2-benzopyrone. The PMN was also migrated by rIL-8. This activity of PMN was inhibited by addition of anti-rIL-8 pAb. These results suggested that chemotactic activity of canine PMN may be mainly mediated by IL-8-like factor(s) produced from PBMC treated with 1,2-benzopyrone.

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1,2-benzopyrone이 개 말초혈액 백혈구의 유주활성에 미치는 영향

김지현 · 강지훈 · 양만표[1]

충북대학교 수의과대학 수의학과 및 동물의학연구소

요 약: 개 말초혈액 백혈구의 유주성에 있어서 1,2-benzopyrone의 효과를 검토하였다. 백혈구의 유주성은 Boyden chamber 변법으로 측정하였다. 1.2-benzopyrone 그 자체는 PMN과 PBMC의 유주성에 직접적인 효과를 보이지 않았다. PMN과 PBMC는 1,2-benzopyrone으로 배양한 PMN의 배양상층액에 대해서도 유주활성을 보이지 않았다. 그러나 1,2-benzopyrone으로 배양한 PBMC의 배양상층액은 PBMC에 대해서는 유주활성을 보이지 않았으나 PMN에 대해서는 현저한 유주활성을 나타내었다. 또한 유주인자인 IL-8에 의한 PMN의 유주활성 측정 결과도 1,2-benzopyrone으로 배양한 PBMC의 배양상층액의 그것과 유사하였다. 이러한 유주활성은 anti-IL-8 pAb를 처리했을 때 PMN의 유주활성이 억제되어, 본 유주활성은 PBMC에서 분비되는 IL-8일 것으로 강하게 시사되었다. 이상의 결과로부터, 1,2-benzopyrone은 개 말초혈액 PMN의 유주성에 대하여 면역자극 작용을 가지고 있으며, 이것은 1,2-benzopyrone의 자극에 의해 PBMC에서 분비되는 IL-8 様 가용성 물질에 의해 나타나는 것으로 사료되었다.

주요어: 개, 1,2-benzopyrone, 유주활성, 백혈구