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A possible role of phospholipase C and phospholipase D in chemotaxis

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Abstract Cell chemotaxis plays a role in many cellular or individual actions suited to accomplishing a variety of purposes. These actions include activities of *bacteria* and *Protista*, cellular *slime molds'* migration to food, starvation-induced aggregation of cellular *slime molds* and mobilization of immunocytes to infectious or inflammatory sites in higher order animals. Macrophages, neutrophils, C3H10T1/2 clone 8 (10T1/2 (SIGMA, USA)) cells, etc. are chemotactic cells. Macrophages and neutrophils are terminally differentiated cells, while 10T1/2 cells are undifferentiated mesenchymal cells. However, currently there is almost no research in which chemotaxis of terminally differentiated cells and undifferentiated mesenchymal cells are comparatively evaluated. Therefore, in this article, the effects of phospholipase C (SIGMA, USA) or phospholipase D (SIGMA, USA) on chemotaxis were evaluated using these 3 types of cells in different differentiation states. The chemotaxis of each cell was evaluated using D609 (SIGMA, USA) and ET-18-OCH_3 (SIGMA, USA) (which are phospholipase C inhibitors), and suramin (SIGMA, USA) and D-erythro-Sphingosine (SIGMA, USA) (which are phospholipase D inhibitors). As chemotactic factors, ZAS was used for macrophages and neutrophils, and platelet-derived growth factor (PDGF) was used for 10T1/2 cells. The chemotaxis of each cell was assessed using 96-hole chemotaxis chambers. Firstly, chemotactic factors were added only to the lower chambers, and chemotaxis was then measured. In macrophages, D609, ET-18-OCH_3, suramin and D-erythro-Sphingosine significantly inhibited chemotactic activity. In neutrophils and 10T1/2 cells, D609 and ET-18-OCH_3 significantly inhibited chemotactic activity. To study these inhibiting mechanisms, further evaluation was performed. ET-18-OCH_3 significantly and dose-dependently inhibited random migration when chemotactic factors were added to both upper and lower chambers. However, ET-18-OCH_3 did not significantly affect chemokinesis where no chemotactic factor had been added. Secondly, in 10T1/2 cells, D609 significantly reduced random migration and chemokinetics, but ET-18-OCH_3 did not significantly change random migration and chemokinesis, even though it slightly reduced them.

Key words
10T1/2,
Chemotaxis,
Phospholipase,
Neutrophil

Introduction

Neutrophils are commonly the first cells of the immune system to encounter invaders such as bacteria and fungi, and are components of innate or natural immune mechanisms. The neutrophil response to infection *in vivo* is initiated by adhesion to vascular endothelial cells, and progresses to directed cell migration into the extravascular tissue space. The migration of macrophages results in phagocytosis and intracellular killing of the invading micro-organisms by generation of bactericidal reactive oxygen species derived from the superoxide anion

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radical. Indeed, neutrophils are essential for controlling most infections, and are mediators of inflammation. The defense function of neutrophils against infection is important for protection of the body from infectious diseases. Inflammatory response is a biological defense reaction advantageous for the body. Particularly, most inflammatory reactions associated with invasion by pathogens are induced to protect the body from pathogens, and thus, complete control of inflammatory reactions by, for example, use of anti-inflammatory drugs is not desirable. Therefore, elucidation of interactions between neutrophil function and exogenous drugs or many endogenous factors is of therapeutic importance. During the process from cytogenesis to morphogenesis, individual cells have polarity that determines their fate, and form various tissues and organs. Determination of cellular polarity is the initial step of determination of the fate of the cells. Cells retain polarity even after formation of the body, and maintain various cellular properties. During morphogenesis, cells with polarity migrate for a long distance in a specific direction, and form an organ at the specific site and time. Determination of cellular polarity and specific migration of cells are regulated by strict signals. Cellular chemotaxis is involved in many purposive movements at the cellular and individual levels such as behavior of bacteria and protozoan, migration of cellular slime mold for food, cell aggregation due to starvation, and immobilization of immune cells to infection and inflammatory sites in higher animals. Before migration of cells, extracellular chemotactic factors detect temperature difference and potentiate intracellular signals on the side of higher concentration, the cell membrane extends at the leading edge, and intracellular organelles such as the nucleus move forward, causing dissociation of the uropod membrane from the extracellular matrix. Phospholipase C is α-toxin of Clostridium welchi, and is also β- and γ-toxins of Clostridium oedematiens. Phospholipase C is an enzyme that catalyzes hydrolysis of phosphatidylcholine (and other phospholipids) to choline phosphate and 1,2-diacetylglycerol, and it also acts on sphingomyelin. It is a key enzyme of synthesis of inositol 1,4,5-thriphosphate. Phospholipase D is an enzyme that produces choline and phosphatidic acid from phosphatidylcholine by hydrolysis, and it also acts on other phosphatidyl esters. Like amoebae of Dictyostelium discoideum (Funamoto *et al.*, 2002; Iijima and Devreotes, 2002), normal blood neutrophils polymerize actin in lamellae at the leading edge, or pseudopod, in a process that depends upon activity of phosphatidylinositol (PI) 3'-kinases (PI3Ks) and activation of Rho GTPases by PI3,4,5-trisphosphate (PI[3,4,5]P_3) (Benard *et al.*, 1999), a lipid product of PI3Ks. However, there has been no study that simultaneously investigated terminal differentiated cells and undifferentiated mesenchymal cells. The mouse fibroblastic cell line, 10T1/2 established from an early mouse embryo, has the pluripotent activity to differentiate into myotubes, adipocytes and chondrocytes when treated with 5-azacytidine. This cell line has been used to study the mechanism of the differentiation from undifferentiated mesenchymal cells into myotubes and adipocytes. However, the possibility that 10T1/2 cells could be induced to differentiate into osteoblasts has not been reported. Both neutrophils and 10T1/2 exhibit chemotaxis. However, neutrophils are terminal differentiated cells, while 10T1/2 are undifferentiated mesenchymal cells.

In this study, we investigated the roles of inhibitors of phospholipases C and D in the mechanism of chemotaxis in these different types of cells: neutrophils and 10T1/2.

Materials and methods

1. Isolation of macrophages and neutrophil from the peritoneum

The protocol employed here meets the guidelines of the Japanese Society for Pharmacology. All efforts were made to minimize animal suffering and to reduce the number of animals used. Macrophages were isolated by peritoneal lavage from adult male Wistar rats weighing 200–250 g, 4 days after injection of 20 m*l* of 1% glycogen. Neutrophil were isolated by peritoneal lavage from adult male Wistar rats weighing 200–250 g, 4 hours after injection of 20 m*l* of 1% glycogen. Lavage was performed by washing the peritoneal cavity with 100 m*l* of ice-cold phosphate buffered saline (PBS, pH 7.2) supplemented with 20 U/m*l* heparin and 1 mM EDTA. Care was taken not to cause internal bleeding while collecting macrophages in the exudate. For chemotaxis and flow cytometry assays, washed macrophages in Dulbecco's modified eagle medium (DMEM) were allowed to adhere to plastic dishes for 30 min at 37°C in humidified atmosphere containing 5% CO_2. They were then resuspended in DMEM. The purity of the adherent cells exceeded 95% as determined

Table 1 Chemoattractant

	Chemotaxis	Random migration	Chemokinesis
Upper	−	+	−
Lower	+	+	−

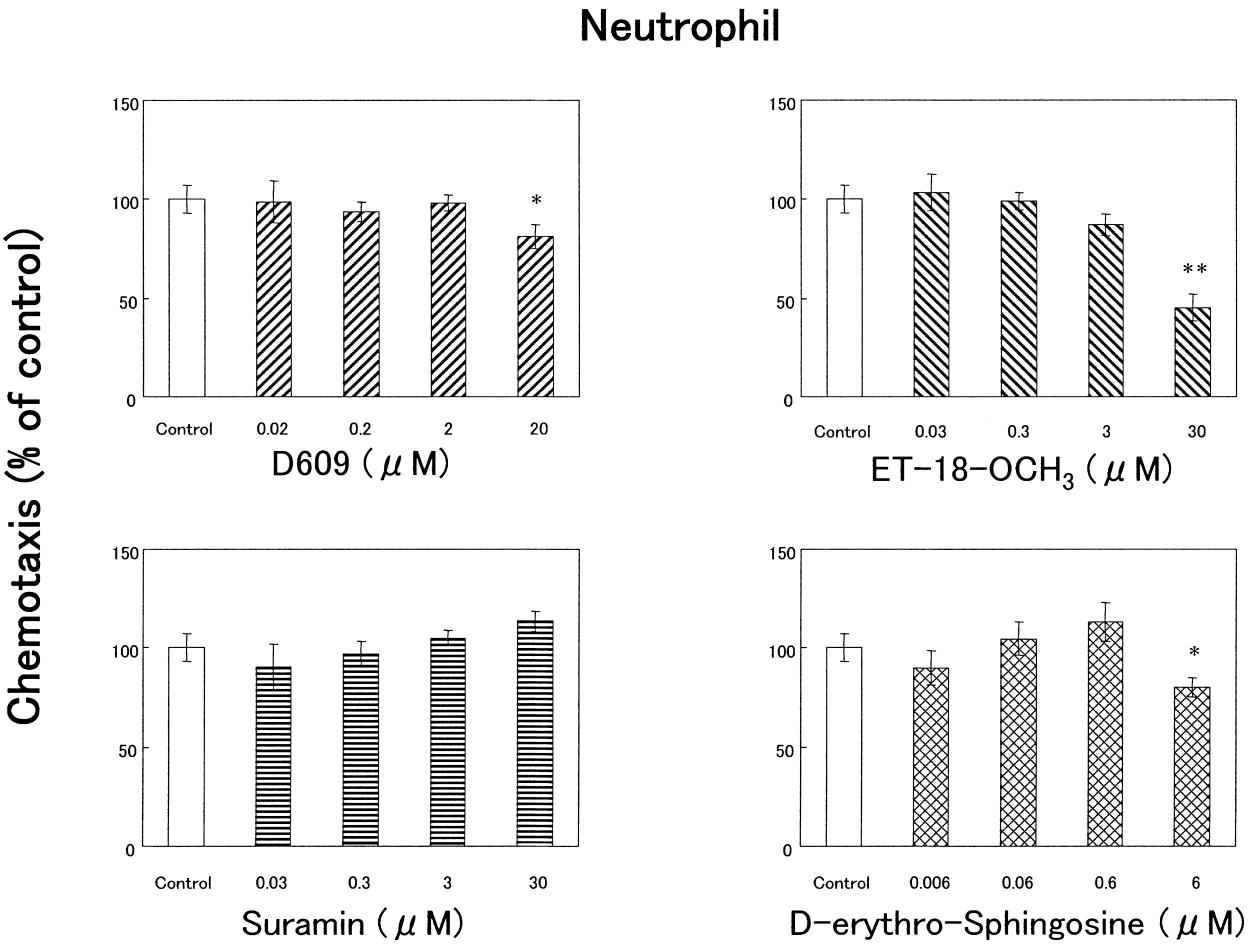

Fig. 1 Drugs inhibit neutrophil chemotaxis towards zymosan-activated serum. Neutrophil and drugs were placed in the upper well, with zymosan-activated serum in the lower well, followed by incubation for 1 hour. The data from 4 individual animals are shown. *$P<0.05$; **$P<0.01$, when compared with untreated neutrophil.

by immunostaining with anti-ED antibody (a macrophage marker).

2. Cell culture

The mouse 10T1/2 (Riken Gene Bank, Tokyo, Japan) was maintained in RPMI-1640 supplemented with 10% FBS, 100 units/m*l* of penicillin and 100μg/m*l* of streptomycin.

3. Chemotaxis assays

Chemotaxis assays were performed as previously described. In brief, zymosan-activated serum, prepared by a method reported elsewhere, was diluted to 5% in medium and placed in the lower compartment of 96-well microchemotaxis chambers as the stimulant in macrophages and neutrophil. However, In brief,

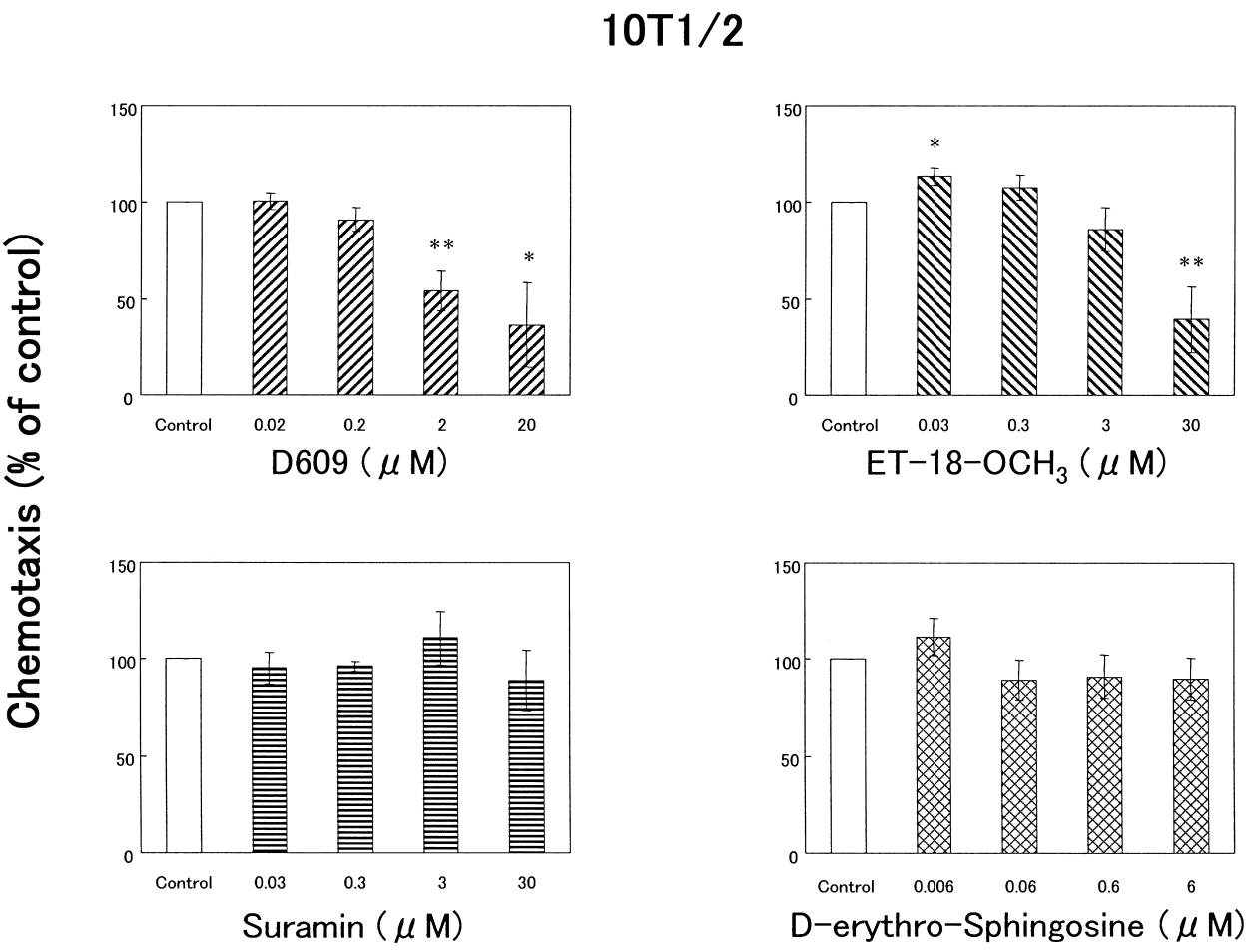

Fig. 2 Drugs inhibit 10T1/2 chemotaxis towards platelet-derived growth factor. 10T1/2 and drugs were placed in the upper well, with platelet-derived growth factor in the lower well, followed by incubation for 6.5 hours. The data from 4 individual animals are shown. *$P<0.05$; **$P<0.01$, when compared with untreated 10T1/2.

platelet-derived growth factor, prepared by a method reported elsewhere, was diluted to 5% in medium and placed in the lower compartment of 96-well microchemotaxis chambers as the stimulant in 10T1/2. A polycarbonate filter with 5μm pores was placed on the wells in case of macrophages and neutrophil, and 8μm pores was placed on the wells in 10T1/2. Experiments were initiated by the addition of the mixture containing macrophages (100μl, 1.4×10^5/well) with drugs to the upper compartment of the chamber. On the other hand, experiments were initiated by the addition of the mixture containing neutrophil (100μl, 2×10^5/well) with drugs to the upper compartment of the chamber. In the meantime, experiments were initiated by the addition of the mixture containing 10T1/2 (100μl, 1×10^5/well) with drugs to the upper compartment of the chamber. After 1.5 hours, the cells on the filter were fixed with methanol and stained with a Diff-Quick in macrophages. In contrast, after 1 hour, the cells on the filter were fixed with methanol and stained with a Diff-Quick in neutrophil. On the other hand, after 6.5 hours, the cells on the filter were fixed with methanol and stained with a Diff-Quick in 10T1/2. The upper side of the filter was then scraped free of cells. The number of cells that migrated to the lower side was determined by measuring optical densities at 595 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA)[1]. Table 1 shows the difference in chemotaxis, random migration and chemokinesis.

4. Data analyses

The results were all expressed as the mean $\pm$ S.E. and the statistical significance was determined by the unpaired-*t*-test followed by estimation of the

Table 2

Drugs (μM)	Chemotaxis (% of control)
Control	100.0 ± 4.3
D609 (20)	54.1 ± 10.2**
ET-18 (30)	73.2 ± 8.14*
Suramin (30)	73.9 ± 8.3*
D-erythro-Sphingosine (6)	72.8 ± 9.9*

Macrophages were allowed to adhere to plates for 2 hours. Adherent macrophages were incubated with drugs containing ZAS at 10μg/m*l* for 3.5 or 24 hours (left and middle column). Adherent macrophages were preincubated with drugs for 24 hours, followed by incubation with ZAS at 10μg/m*l* for 24 hours (right column). The data from 3 to 5 individual animals are shown. *$P<0.05$; **$P<0.01$, when compared with macrophages treated with ZAS alone.

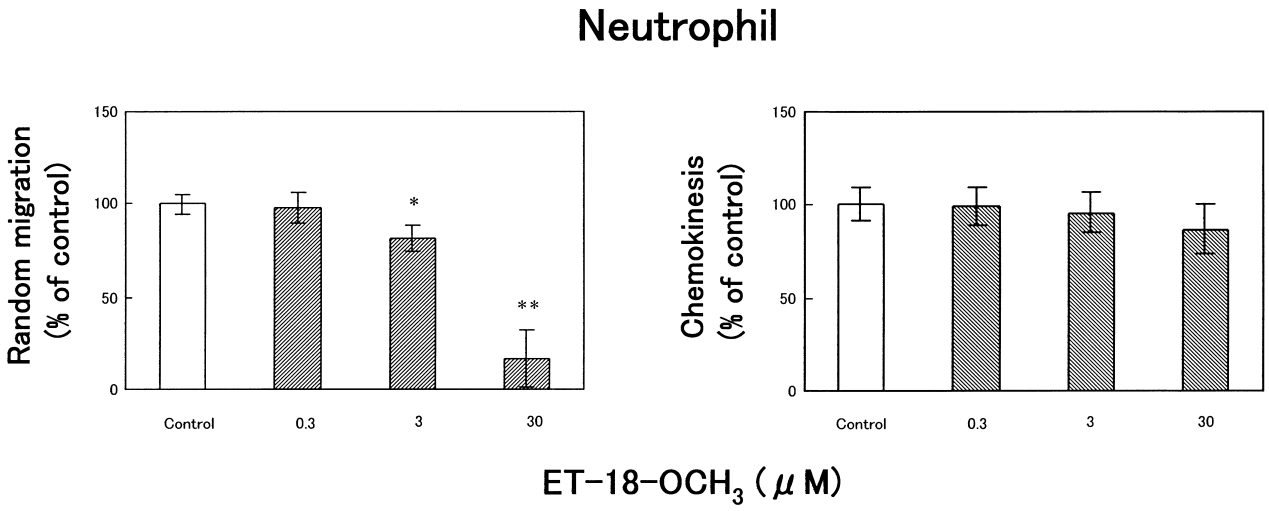

Fig. 3 Drugs inhibit neutrophil random migration towards zymosan-activated serum. Neutrophil and drugs were placed in the upper well, with medium in the lower well, followed by incubation for 1 hour. Drugs inhibit neutrophil chemokinesis towards zymosan-activated serum. Neutrophil and drugs and zymosan-activated serum were placed in the upper well, with zymosan-activated serum in the lower well, followed by incubation for 1 hour. The data from 4 individual animals are shown. *$P<0.05$; **$P<0.01$, when compared with untreated neutrophil.

least significant difference.

Results

I. Chemotaxis

In neutrophils, D609 significantly decreased at 20μM. Similarly, ET-18-OCH_3 significantly decreased at 30μM. However, suramin did not cause change at any concentration. D-erythro-Sphingosine significantly decreased at 6μM (Fig. 1). In 10T1/2, significant decreases were observed at 2 and 20μM. In the presence of ET-18-OCH_3, significant increase was caused at 0.03μM, but marked decrease was caused at 30μM. In contrast, no change was observed in the presence of suramin at any concentration. Moreover D-erythro-Sphingosine did not cause change at any concentration (Fig. 2). Similarly, in macrophages, significant decrease was caused by 20μM D609. Significant decrease was also caused by 30μM ET-18-OCH_3. Suramin markedly decreased chemotaxis at 30μM. Similarly, 6μM D-erythro-Sphingosine significantly decreased chemotaxis (Table. 2).

II. Random migration-Chemokinesis

Inhibition of chemotaxis by D609 and ET-18-OCH_3 suggested that cells became unable to recognize the

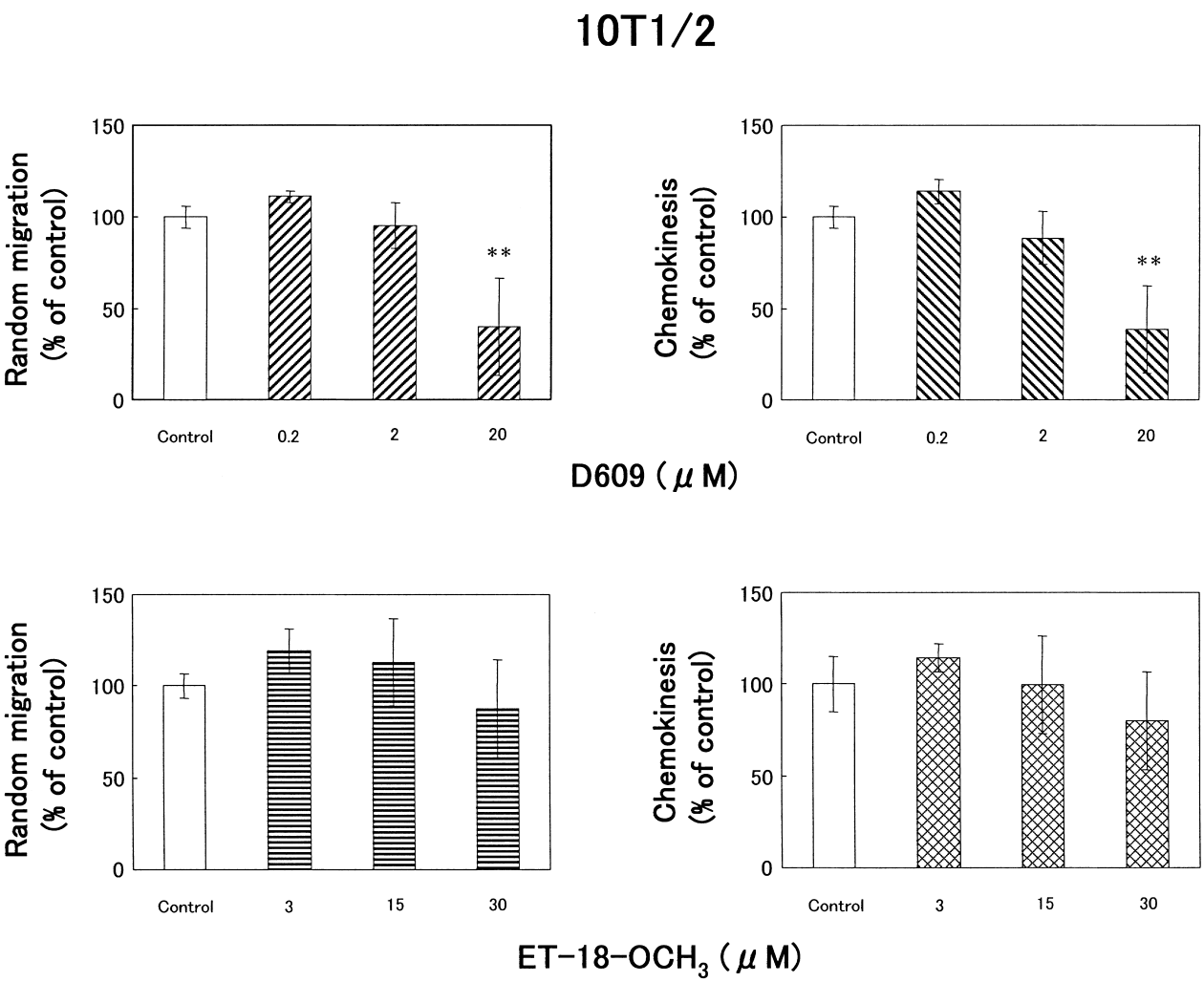

Fig. 4 Drugs inhibit 10T1/2 random migration towards platelet-derived growth factor. Neutrophil and drugs were placed in the upper well, with medium in the lower well, followed by incubation for 1 hour. Drugs inhibit neutrophil chemokinesis towards platelet-derived growth factor. Neutrophil and drugs and platelet-derived growth factor were placed in the upper well, with platelet-derived growth factor in the lower well, followed by incubation for 6.5 hours. The data from 4 individual animals are shown. $^{**}P<0.01$, when compared with untreated neutrophil.

direction or move. To investigate these inhibition mechanisms, we preceded the investigation to random migration and chemokinesis. In neutrophils, we investigated random migration and chemokinesis in the presence of ET-18-OCH_3. Random migration significantly decreased at 3 and 30μM. However, chemotaxis did not change at any concentration (Fig. 3). Next, 10T1/2 was reacted with D609 and ET-18-CH_3. Random migration significantly decreased at 30μM by D609. Similarly, chemokinesis significantly decreased at 30μM. However, in the presence of ET-18-OCH_3, no significant changes were observed in random migration or chemokinesis at any concentration (Fig. 4).

Discussion

Differences in chemotaxis of the same cells caused by the agents were investigated. In neutrophils, chemotaxis was markedly inhibited by phospholipase C inhibitors, D609 and ET-18-OCH_3, and significantly inhibited by a phospholipase D inhibitor, D-erythro-Sphingosine. In 10T1/2, marked decreases by D609 and ET-18-OCH_3 were observed, but suramin and D-erythro-Sphingosine did not change chemotaxis. In macrophages, all agents significantly decreased chemotaxis. Next, differences caused by the same agent among the cell types were investigated. As for phospholipase C inhibitors, D609

significantly decreased chemotaxis in all cell types, and ET-18-OCH_3 also markedly decreased chemotaxis in all cell types. The phospholipase C inhibitors significantly inhibited chemotaxis of all cell types. As for phospholipase D inhibitor, suramin tended to decrease chemotaxis of only macrophages, while D-erythro-Sphingosine inhibited chemotaxis of neutrophils and macrophages. The difference between the two agents may be due to that D-erythro-Sphingosine is not only a phospholipase D inhibitor but also inhibitor of protein kinase and phospholipase A_2. The above findings confirmed that D609 and ET-18-OCH_3 markedly inhibited chemotaxis of neutrophils and 10T1/2. Since phospholipase C inhibitors, D609 and ET-18-OCH_3, markedly inhibited chemotaxis, we preceded the study to investigation of random migration and chemokinesis to investigate the inhibition mechanism. In the investigation of neutrophils in the presence of ET-18-OCH_3, random migration was significantly inhibited in a concentration-dependent manner, but no marked change was observed in chemokinesis. In 10T1/2, random migration and chemokinesis were investigated in the presence of D609 and ET-18-OCH_3. Random migration and chemokinesis significantly decreased in the presence of D609, but decreases were only slight in the presence of ET-18-OCH_3 without significant differences. D609 is phosphatidylcholine-specific phospholipase C inhibitor, while ET-18-OCH_3 is phosphatidylinositol-specific phospholipase C inhibitor, suggesting that specificity to choline is involved in the cell movement, while specificity to inositol is involved in recognition of direction of cells. In this study, we analyzed the relationship between phospholipases C and D and chemotaxis, but phospholipases C and D have been reported to affect not only chemotaxis but also other functions. For example, Pseudomonas aeruginosa hemolytic phospholipase C has been reported to inhibit respiratory burst activity of neutrophils in an investigation of O_2 productivity[2]. In addition, phospholipase D has been shown to be activated by Rho-binding protein, a member of the membrane-bound low-molecular weight GTP family[3]. Furthermore, RhoA is able to reform GTPγS-dependent phospholipase D activity in non-cellular fraction of human neutrophils in the presence of 50 kDa factor present in the cytoplasm[4]. Regulation of differentiation by phospholipase D activity, regulation of degranulation by genistein and EGCG in FMLP-stimulated neutrophils[5], and decreased phospholipase D activity in incomplete oxidation metabolism in neutrophils in polycythemia vera have been shown[6]. These reports suggested that activation of phospholipases C and D are not only involved in chemotaxis but also plays some roles in various functions. Phospholipases C and D were not involved in neutrophil chemotaxis in some studies. For example, it has been reported that the pleckstrin homology domain of AKT protein kinase is expressed in neutrophil chemotaxis as a signal probe of intracellular response[7], Rac and Cdc42 play obvious roles in regulation of PI(3,4,5)P_3 and polarity during neutrophil chemotaxis[8], and calcium-dependent protease, calpain, regulates neutrophil chemotaxis in chemotaxis assay[9]. In this study, we clarified the importance of phospholipases C and D in macrophages, neutrophils, and 10T1/2, but possibility remains that it is not based on the past reports. More extensive investigation of the roles of factors related to chemotaxis is necessary. We used PDGFα as the chemotactic factor for 10T1/2. It has been reported that PDGFα promoted growth of basal cells in cell proliferation assay[10], suggesting that PDGF not only induces chemotaxis but also is related to growth of 10T1/2. In another report, a mouse non-osteogenic multipotential cell line, 10T1/2, was induced to differentiate to osteoblasts by recombinant human morphogenesis protein 2 in northern blot analysis, and this cell line is also used in studies other than study of chemotaxis[11]. The above findings suggested that phospholipases C and D are involved in chemotaxis regardless of the cell types, but the effects of phospholipases C and D are different. In addition, phosphatidylcholine-specific phospholipase C and phophatidylinositol-specific phospholipase D may play different roles in chemotaxis of 10T1/2. Finally this study clarified the initial stage of the healing process of inflammation of tissue such as the gingiva and oral mucosa in children, providing information useful for establishing principles in clinical pedodontics.

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