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1α,25-Dihydroxyvitamin D_3 Decreased ICAM-1 and ELAM-1 Expressions on Pulmonary Microvascular Endothelial Cells and Neutrophil Motivation

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Preincubation of pulmonary microvascular endothelial cells (PMVECs) with platelet activating factor (PAF) for 3.5 h increased the adhesion rate of polymorphonuclear leukocytes (PMNs) to PMVECs from 57.3 to 72.8% ($P < 0.01$). Coincubations of 1α,25-dihydroxyvitamin D_3 (VD) or MC903 (VD analog) decreased the ability of PAF to increase PMN–PMVEC adhesion rate significantly. Preincubation of PMNs with PAF also increased PMN–PMVEC adhesion rate. Coincubation of VD but not MC903 blocked PAF-induced adherence of PMNs to PMVECs. Both VD and MC903 decreased PAF-increased expression of ICAM-1 on PMVECs, PMN chemotaxis to zymosan activated serum and histamine, and PMN aggregation induced by PAF significantly. VD but not MC903 decreased the expression of ELAM-1 on PMVECs. VD and MC903 did not affect PAF-induced release of acid phosphatase from PMNs. The results suggest that VD may be a good drug to inhibit PMN motivation by acting on both ECs and PMNs.

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INTRODUCTION

Polymorphonuclear neutrophils (PMNs) play a key role in the inflammatory response underlying many diseases. PMNs are capable of chemotaxis, phagocytosis, oxygen free-radical production and degranulation in response to a variety of stimuli. Overstimulation of PMNs can result in host tissue injury. Inhibition of PMN functions may be beneficial to inflammatory diseases such as systemic inflammatory reaction syndrome [1]. During inflammation, leukocytes may adhere firmly on microvascular endothelial cells (ECs), whereupon they project pseudopodia and migrate across the endothelial monolayer into traumatized interstitia through diapedesis. The localized adhesion of leukocytes to ECs is mediated at least partly by adhesion molecules on leukocytes and their complementary molecules on ECs such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin (ELAM-1) [2].

Substantial evidence is accumulating that calcitriol plays an important role in the immune system. $1\alpha,25(OH)_2D_3$(VD) decreases total T-cells and CD4+:CD8+ ratios [3]. VD increases cytotoxicity and exocytosis in lymphokine-activated killer cells [4]. Calcitriol inhibits the production of interleukin-2, interferon-γ and granulocyte macrophage colony stimulating factor [5, 6]. Studies on the effects of calcitriol on the production and their action of interleukin-1 and tumor necrosis factor have shown both stimulation and inhibition [5, 7–11].

The study of the effects of VD on PMNs focuses on the proliferation of PMNs. Whether VD influences the functions of mature PMNs and whether mature PMNs express VD receptor are unknown.

MATERIALS AND METHODS

Reagents

$1\alpha,25(OH)_2D_3$ and its analog, MC903, were gifts from Leo Pharmaceutical Products Ltd. Monoclonal antibodies 1.2B6 (anti-human ELAM-1) and 6.5 (anti-human ICAM-1) were gifts from Dorian O. Haskard, Royal Postgraduate Medical School, University of

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London. Dulbecco's Modified Eagle Medium (DMEM) and dextran 500 were purchased from Sigma (St Louis, MO). Other reagents were purchased from Shanghai Chemical Reagent Co.

Zymosan activated serum (ZAS)

Zymosan A was suspended in normal rat serum at a concentration of 2 mg/ml and incubated at 37°C for 30 min. The serum was then centrifuged at 1500 *g* for 10 min. The supernatant was divided into aliquots and kept frozen at −20°C until use.

Isolation of the endothelial cells

Sprague–Dawley rats weighing 80–100 g were anesthetized with urethane (2 g/kg body weight) and heparinized (1000 units per animal) intraperitoneally. The animals were exsanguinated by cutting the bilateral carotid arteries. The blood remaining in the pulmonary vascular bed was washed out with Hanks' solution. The lungs were isolated. The tissues of the lung surface or edge were cut into pieces of 1 × 1 × 1.5 mm separately. Ten pieces were placed into a flask with 45 cm^2 bottom surface and cultured in DMEM supplemented with 20% fetal bovine serum. No antibiotics, growth factors and extracellular matrix proteins were added. After 60 h culture, the tissues were discarded and medium partially changed. The flask contained only ECs and blood cells. The cells were subcultured with 0.08% trypsin in D-Hanks' solution between the 6th and 10th days. The cells were identified to be pulmonary microvascular endothelial cells (PMVECs) according to morphological and functional criteria [12].

Measurement of polymorphonuclear leukocyte (PMN) adhesion to PMVECs

PMNs were isolated from heparinized rat blood with dextran sedimentation and centrifugation on Ficoll–Hypaque discontinuous density as previously reported [13]. Cell viability with the trypan blue exclusion test was more than 99%. PMN adhesion rate = (PMNs adhered/PMNs added) × 100%. PMN adhesion rate was measured by the following two methods. PMNs in Hanks' solution were added to PMVEC monolayers pretreated as experimental protocol. After incubation at 37°C in 5% CO_2 and 95% air for 30 min, PMVEC monolayers with adherent PMNs were washed gently with the culture medium. First, PMN adhesion rate was calculated from the counting difference between PMNs added and aspirated. Second, PMNs adhesion rate was calculated under a phase-contrast microscope. The total adhered PMNs = (the area of one culture well/the area of one vision) × the adhered PMNs of one vision. The adhesion rates measured by the two methods were not significantly different. The average adherence rate obtained from the two methods is presented in the results.

ELISA for the expression of ICAM-1 and ELAM-1 on PMVECs

PMVECs were plated in 96-well microtiter plates at a concentration of 4×10^4 cells and were preincubated with culture media alone, PAF (10^{-8} mol/l), PAF plus VD (2.2×10^{-9} mol/l), or PAF plus MC903 (2.2×10^{-9} mol/l) for 4 h at 37°C. Culture supernatants (100 μl) contained either monoclonal antibodies 1.2B6 or 6.5B%. The plates were incubated at 37°C for 30 min. After washing, 100 μl peroxidase-conjugated goat anti-mouse IgG, diluted 1:500, were added to each well and the plates were incubated for 30 min. The plates were washed again. *o*-Phenylenediamine (100 μl) and 30 μg H_2O_2 in 100 μl citrate–phosphate buffer (pH 5.0) were added. The plates were incubated at 37°C for 30 min. The chromogenic reaction was stopped with 100 μl 2N H_2SO_4 and the plates read spectrophotometrically at 492 nm on an ELISA reader (DG 3022A, East China Electron Tube Factory).

The effects of VD and MC903 on PMN–PMVEC adherence

(1) The ECs were cultured to confluent monolayer on 96-well plates and preincubated for 3.5 h with culture media alone, PAF (10^{-8} mol/l), VD (2.2×10^{-9} mol/l) plus PAF, or PAF plus MC903 (2.2×10^{-9} mol/l). PMNs (1×10^5 cells/well) were added and incubated for 30 min. The adhesion rate was measured as described elsewhere.

(2) PMNs were preincubated in the same way as PMVECs. After preincubation, PMNs were added to untreated PMVECs and adhesion rate was measured.

Chemotaxis assay

PMN chemotaxis was measured as described by Nelson [14]. PMNs were suspended in cultured medium contained VD (10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} mol/l) or MC903 (10^{-11}, 10^{-10}, 10^{-9} or 10^{-8} mol/l). PMNs (2.5×10^5)/well were added to agarose holes and incubated at 37°C in 5% CO_2 for 18 h. The cells were dehydrated in 75% ethanol and stained by Wright's method. The distances of the migration front towards ZAS well or histamine (8.7×10^{-8} mol/l) were measured with an internal microscope micrometer.

PMN aggregation

PMNs were preincubated with VD (4×10^{-9} or 4×10^{-11} mol/l), MC903 (10^{-9} mol/l) or medium alone for 4 h. The PMN aggregation caused by PAF was determined on a PPP automatically balanced platelet aggregator (Shanghai Keda Apparatus Factory). PAF (50 μl, 10^{-8} mol/l) caused maximal aggregation. PAF (30 μl, 10^{-8} mol/l) as used to measure the samples. PMN aggregation(%) = PMN aggregation of sample × 100%/maximal PMN aggregation.

Statistical analyses

Data are expressed as the mean ± SEM. Statistical analyses were performed by unpaired Student's *t*-tests.

RESULTS

The effects of VD and MC on PMN–PMVEC adhesion

Stimulation of PMVEC with PAF for 3.5 h resulted in a significant increase in PMN–PMVEC adhesion rate from 57.3 to 72.8% ($P < 0.01$). In the presence of VD or MC903, the adhesion rate decreased by 18.1 and 14.3%, respectively ($P < 0.01$, Fig. 1). Preincubation of PMNs with PAF also increased the PMN–PMVEC adhesion rate significantly ($P < 0.01$). Addition of VD but not MC903 blocked PAF-induced PMN–PMVEC adhesion (Fig. 2).

Expression of ELAM-1 and ICAM-1 on endothelial cells

Normal PMVECs did not express ELAM-1. The expression of ELAM-1 was increased after treatment with PAF. VD decreased the expression of ELAM-1 induced by PAF. MC903 did not affect the expression of ELAM-1 (Fig. 3).

PAF increased ICAM-1 expression. VD but not MC903 decreased PAF-increased ICAM-1 expression (Fig. 4).

PMN chemotaxis

Migration distances of control PMNs to ZAS and histamine were 344 ± 23 and 270 ± 7 mm, respectively. Migration distances of PMNs treated with 10^{-10}, 10^{-11} mol/l VD and 10^{-9} mol/l MC903 to ZAS were 177 ± 7, 132 ± 18 and 120 ± 2 mm, respectively, significantly lower than control ($P < 0.01$). VD (10^{-10} mol/l) and 10^{-9} and 10^{-10} mol/l MC903 decreased histamine induced PMN chemotaxis ($P < 0.01$). Other concentrations of VD and MC903 did not decrease PMN chemotaxis to ZAS or histamine significantly.

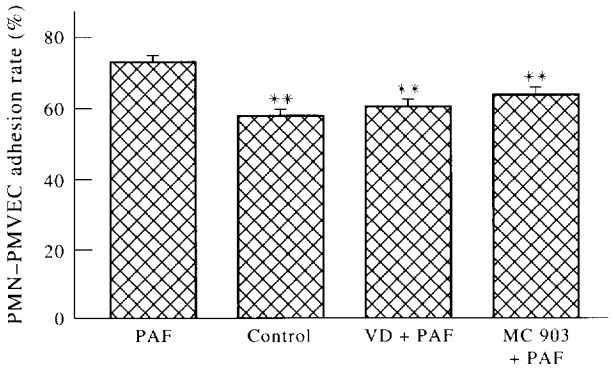

Fig. 1. $1\alpha,25(OH)_2D_3$ (VD) decreased the adhesion rate of PMNs to pulmonary microvascular endothelial cells (PMVECs) pretreated with platelet activating factor (PAF) for 3.5 h. **$P < 0.01$ vs PAF.

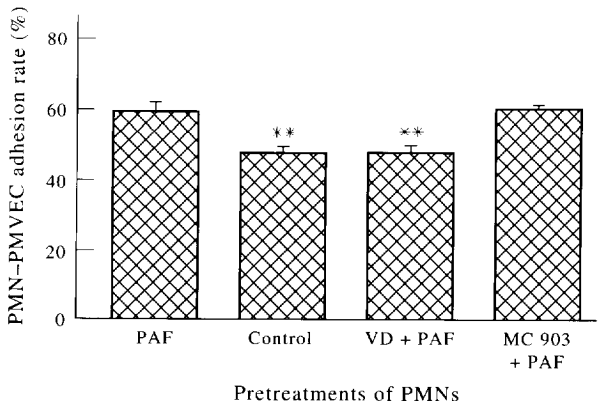

Fig. 2. $1\alpha,25(OH)_2D_3$ (VD) decreased the adhesion rate of PMNs pretreated with platelet activating factor (PAF) for 3.5 h to untreated pulmonary microvascular endothelial cells (PMVECs). *$P < 0.05$, **$P < 0.01$ vs PAF.

PMN aggregation

The aggregation of PMNs treated with 4×10^{-9}, 4×10^{-11} mol/l VD or 10^{-9} mol/l MC903 were 19.2, 22.2 and 16.4% lower than control (Fig. 5), respectively.

The release of acid phosphatase

VD and MC903 (10^{-11}–10^{-8} mol/l) did not decrease PAF-induced acid phosphatase from PMNs.

DISCUSSION

VD decreased PMN proliferation [15] and PMN accumulation in psoriasis [16]. We recently found that VD decreased the mortalities of rat and mouse induced by endotoxin from 80 and 70% to 14 and 10%, respectively. VD decreased pulmonary vascular permeability and lung injury caused by endotoxin, PLA_2 and PAF in intact animals, isolated perfused lungs and cultured endothelial monolayers (unpublished data).

PAF stimulated cultured ECs to become more adherent by the expression of ELAM-1 and ICAM-1 [17]. In this experiment, VD decreased the adherence of PMNs to PAF-stimulated PMVEC while the

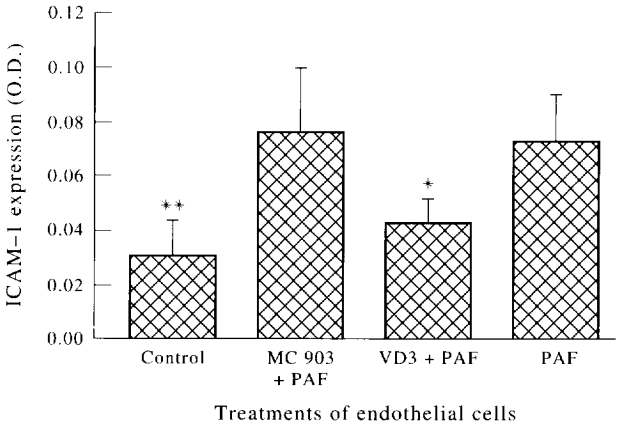

Fig. 3. The effects of $1\alpha,25(OH)_2D_3$ (VD) and MC903 on platelet activating factor (PAF)-induced ICAM-1 expression on pulmonary microvascular endothelial cells. *$P < 0.05$, **$P < 0.01$ vs PAF.

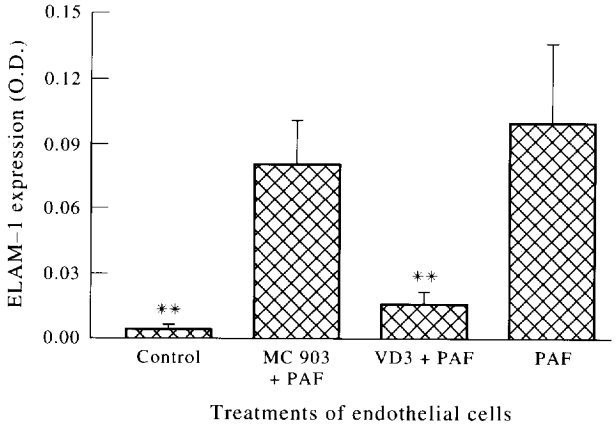

Fig. 4. The effects of $1\alpha,25(OH)_2D_3$ (VD3) and MC903 on platelet activating factor (PAF)-induced ELAM-1 expression on pulmonary microvascular endothelial cells. $^{**}P < 0.01$ vs PAF.

expressions of ICAM-1 and ELAM-1 decreased. VD and MC903 decreased the adherence of PAF-stimulated PMNs to PMVECs, also. Both VD and MC903 decreased PMN migration distance induced by ZAS or histamine. Our results were compatible with the study that VD decreased PMN in skin of psoriasis patients [16]. Both VD and MC903 decreased PMN aggregation significantly. Thus, it is likely that VD and its analogue may inhibit PMN adhesion to and migration across endothelial monolayer where inflammation occurs.

PMNs from patients with hereditary resistance to VD reduced nitroblue tetrazolium dye, and generated superoxide ions and hydrogen peroxide normally [18]. VD incubated with PMNs did not decrease PAF-induced acid phosphatase release. These data suggest that VD may not decrease PMN degranulation.

Glucocorticoids are known steroids that inhibit PMN functions. Our recent *in vivo* studies showed that another steroid, retinoic acid, also decreases the expression of ELAM-1 and ICAM-1 on ECs, PMN–PMVEC adhesion and PMN chemotaxis (unpublished data). Whether the inhibition of PMN function is the common action of all steroids and whether different kind of steroids inhibit PMN functions through a common mechanism need to be investigated.

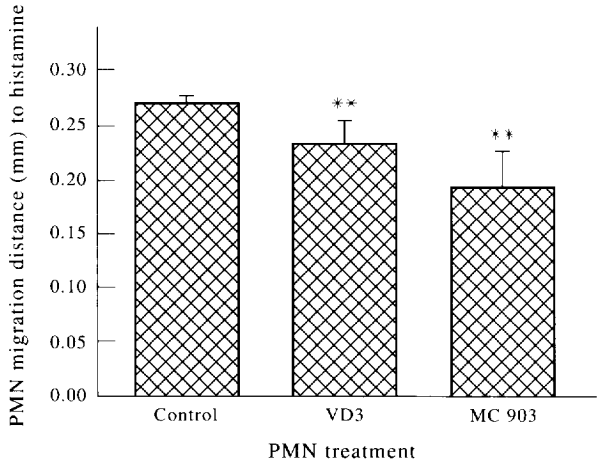

Fig. 5. $1\alpha,25(OH)_2D_3$ (VD3) and MC903 decreased histamine-induced chemotaxis. $^{**}P < 0.01$ vs control.

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