Secretion of soluble-form CD93 from human endothelial progenitor cells

Nobunao IKEWAKI , Tohru SONODA* , Koutarou DOI** , Eiji OHNO***

Abstract

In this study, we examined the secretion of soluble-form CD93 (sCD93) from human endothelial progenitor cells (HEPCs) expressing cell surface CD93 by enzyme-linked immunoassay (EIA) using CD93 monoclonal antibody (mAb) (mNI-11) established in our laboratories. We found that a small amount of sCD93 was spontaneously secreted into the culture supernatants of HEPCs. Furthermore, significantly ($P < 0.01$) enhanced secretion of sCD93 was found in the culture supernatants of HEPCs treated with phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, which was significantly ($P < 0.01$) suppressed by treatment of the cells with the protein kinase (PKC) inhibitor Go6976. Interestingly, depolymerization of the actin filaments in the cells by cytochalasin E (CyE) also significantly ($P < 0.01$) enhanced the sCD93 secretion into the culture supernatants of HEPCs. Taken together, these findings suggest that the sCD93 might serve as a new biomarker for analyzing the biological and immunological functions of HEPCs.

Key words : cytochalasin E, human endothelial progenitor cells, phorbol 12-myristate 13-acetate, soluble-form CD93 molecule.

Introduction

Human CD93 is a heavily *O*-glycosylated type I transmembrane glycoprotein consisting of unique C-type lectin domains (CTLDs) [1,2]. CD93 is mainly expressed on myeloid cells (monocytes and granulocytes), hematopoietic stem cells and endothelial cells[3-5]. In addition, CD93 is also co-expressed on naive T lymphocytes ($CD4^+CD45RA^+$cells) of neonatal umbilical cord blood cells (UCBCs), but not on normal adult peripheral blood cells (PBCs)[6]. In terms of the biological functions, CD93 reportedly plays a critical role in the clearance/exclusion of apoptotic cells[7,8] and in angiogenesis[9].

On the other hand, soluble-form CD93 (sCD93) has also been detected in the human serum/plasma of normal adults[10]. Furthermore, the sCD93 levels in the neonatal umbilical cord blood (UCB) have been shown to be significantly higher than those in normal adult peripheral blood (PB)[11),12)] . Meanwhile, cell surface CD93 is also reportedly shed from activated human myeloid cells (monocytes and neutrophils) in the form of sCD93. In particular, sCD93 shedding/release was detected *in vitro* (culture supernatant) in response to treatment with lipopolysaccharide (LPS), tumor necrosis factor (TNF)- α and phorbol 12-myristate 13-acetate (PMA), the protein kinase C (PKC) activator[10-12].

As described above, although CD93 is a physiologically

Laboratory of Clinical Immunology, Department of Animal Pharmaceutical Science, Kyushu University of Health and Welfare School of Pharmaceutical Sciences , 1714-1 Yoshino-machi, Nobeoka-shi, Miyazaki 882-8508, Japan
*Department of Occupational Therapy, Kyushu University of Health and Welfare School of Health Science, 1714-1 Yoshino-machi, Nobeoka-shi, Miyazaki 882-8508, Japan
**Department of Obstetrics and Gynecology, Miyazaki Prefectural Nobeoka Hospital 2-2-10 Shinkoji, Nobeoka-shi, Miyazaki 882-0835, Japan
***Quality of Life Research Organization, Kyushu University of Health and Welfare 1714-1 Yoshino-machi, Nobeoka-shi, Miyazaki 882-8508, Japan

and immunologically important and unique molecule in immune regulation, the patterns and levels of sCD93 secretion from human endothelial progenitor cells (HEPCs) remain very poorly understood.

In this study, we investigated the secretion levels of sCD93 from HEPCs in response to treatment with several substances such as a PMA and cytochalasin E (CyE), a drug that causes actin filament depolymerization, by an enzyme-linked immunoassay (EIA) using CD93 monoclonal antibody (mAb) (mNI-11)[3,6,12,13] established in our laboratories.

Materials and Methods

Antibodies and reagents

Phycoerythrin (PE)-conjugated or non-conjugated CD93 monoclonal antibody (mAb) (mNI-11) (mouse IgG1)[3,6,12,13] was established in our laboratories. A biotin-conjugated CD93 mAb, X-2 (mouse IgG1), and horseradish peroxidase (HRPOD)-conjugated streptavidin were purchased from Cosmo Bio Co. (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated CD34 mAb (My10) (mouse IgG1) was purchased from BD Biosciences Co. (U.S.A.). PE-conjugated CD49d mAb (BU49) (mouse IgG1) was purchased from Ancell Co. (U.S.A.). An FITC-conjugated CD146 mAb (541-10B2) (mouse IgG1) was purchased from Miltenyi Biotec Co. (U.S.A.). PE-conjugated or FITC-conjugated isotype-matched normal mouse IgG1 was purchased from MBL Co. (Nagoya, Japan). Phorbol 12-myristate 13-acetate (PMA) and a conventional protein kinase C (cPKC) inhibitor (Go6976) were purchased from Cosmo Bio Co. (Tokyo, Japan). Cytochalasin E (CyE) was purchased from Sigma Co. (U.S.A.).

Human endothelial progenitor cells (HEPCs)

Cryopreserved human endothelial progenitor cells (HEPCs) (Z7030001) and growth medium (Z7030003) were obtained from BCH Co. (U.S.A.). The HEPCs were cultured in the growth medium, according to a routine protocol.

Analysis of HEPCs treated/not treated with various substances

HEPCs (2 x 10^5 cells/mL) in growth medium (4 mL) were seeded in culture flasks (Sumitomo Co., Tokyo) and treated/not treated with PMA (100 ng/mL) in the presence or absence of the conventional protein kinase C (cPKC) inhibitor, Go6976 (3.0 μM), for 6 hrs, 10 hrs or 24 hrs in a CO_2 incubator. In an additional experiment, the HEPCs were treated or not treated with cytochalasin E (CyE), a drug that causes actin filament depolymerization (100 ng/mL) under the same conditions. The culture supernatants were collected and analyzed for the presence of soluble-form CD93 (sCD93) by an originally constructed enzyme-linked immunoassay (EIA) system. On the other hand, the morphological changes of the HEPCs treated or not treated with the aforementioned substances were also observed and photos were taken under a phase-contrast microscope.

Flow cytometry

The HEPCs were washed in cold phosphate-buffered saline (PBS) containing 0.1% NaN_3 (hereinafter referred to as the washing buffer) and incubated in washing buffer containing 25% normal goat serum and 1 mg/mL of normal human IgG for 10 min on ice, to block the Fc receptor for IgG. The cells were then incubated with an optimal concentration of PE-conjugated CD93 mAb (mNI-11), FITC-conjugated CD34 mAb (My10), FITC-conjugated CD146 mAb (541-10B2) or PE-conjugated CD49d mAb (BU49) for 40 min at room temperature. A negative control (NC) was set by incubation of the cells with mixed PE-conjugated and FITC-conjugated isotype-matched normal mouse IgG1 under the same conditions. Following a final wash with the washing buffer, the cellular debris was excluded. Thereafter, the percentages of positively stained cells for each mAb were determined as single-color flow-cytometric profiles using a fluorescence activated cell sorter (FACScan) (Becton Dickinson Co.). The percentages of cells showing positive staining for each of the mAbs were calculated for each FACS profile. The experiment was carried out in triplicate.

Detection of soluble-form CD93 (sCD93)

EIA plates (Sumitomo Co., Tokyo) were coated with 500 ng/mL of CD93 mAb (mNI-11) in carbonate-bicarbonate buffer (0.01M $NaCO_3$, 0.035M $NaHCO_3$, pH9.6) for 24 hrs at 4° C. The wells were washed four times with PBS containing 0.05% Tween-20 (PBST) and blocked with PBST containing 2% BSA (BSA-PBST) for 60 min at room temperature. Thereafter, the wells were washed three times with PBST. Culture supernatants (x2 dilution with 0.1% BSA-PBST; 50 μL) and biotin-labeled CD93 mAb (X-2) (x2,500 dilution with 0.1% BSA-PBST; 50 μL) were then added to each well, followed by incubation for 60 min at room temperature with shaking. A recombinant protein of the CD93 molecule (TP306980) (Origene Technologies, Inc.) was added to each well as a positive control or as a CD93-standard. The wells were washed eight times with PBST and HRPOD-conjugated streptavidin (x5,000 dilution with 0.1% BSA-PBST; 100 μL) was added to each well, followed by incubation for 30 min at room temperature with shaking. The wells were then washed 10 times with PBST, and the substrate-chromogen (TMB; Cosmo Bio Co.; 100 μL) was added to each well, followed by incubation for 10 min at room temperature with gentle shaking. The reaction was stopped by the addition of 0.5N-HCl (100 μL), and the optical density (O.D.) was read at 450 nm using a multichannel EIA-microplate reader (TOSOH Co.). The experiment was repeated five times.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The statistical analysis was performed using an unpaired Student *t*-test. Differences at $P < 0.01$ were considered to be significant.

Results and Discussion

First of all, we investigated the expressions of CD93 (hematopoietic progenitor/endothelial cell marker), CD34 (hematopoietic stem cell marker), CD146 (endothelial cell marker) and CD49d (α4 integrin marker) on the HEPCs using a single-color flow-cytometric system. As shown in Figure 1, CD93 (82.4%), CD34 (18.9%) and CD146 (99.3%) were expressed on the HEPCs, while no expression of CD49d (0.9%) was found on the cells. These findings suggest that the HEPCs used in this study had the properties of progenitor-related cells.

Next, we investigated the amounts of soluble-form CD93 (sCD93) secreted by the HEPCs treated or not treated with PMA at 6 hrs, 10 hrs, and 24 hrs, by an originally constructed EIA system using a CD93 mAb (mNI-11). As shown in Figure 2, a small amount of sCD93 was spontaneously secreted by the HEPCs into the culture supernatants at 6 hrs (7.7 ng/mL, on average), 10 hrs (9.5 ng/mL, on average), and 24 hrs (9.6 ng/mL, on average) in the absence of PMA treatment. On the other hand, strongly enhanced secretion of sCD93 was found at 6 hrs (21.5 ng/mL, on average), 10 hrs (24.8 ng/mL, on average), and 24 hrs (34.5 ng/mL, on average) in the culture supernatants of the HEPCs treated with PMA. These data indicate that secretion of sCD93 was significantly enhanced by PMA treatment even from an early phase (6 hrs), and that PMA treatment significantly ($P < 0.01$) enhanced the amount of sCD93 secreted by the HEPCs into the culture supernatants of HEPCs as compared to the amount secreted by the control HEPCs (not treated with PMA). Furthermore, the amounts of sCD93 secreted into the culture supernatants by the HEPCs treated with PMA in the presence of the conventional protein kinase C (cPKC) inhibitor Go6976 (3.0 μM) were 11.5 ng/mL, on average, at 6 hrs, 12.6 ng/mL, on average, at 10 hrs, 16.2 ng/mL, on average, at 24 hrs (Fig. 2); namely, the enhanced secretion of sCD93 by the HEPCs induced by PMA was significantly ($P < 0.01$) suppressed by concomitant treatment of the cells with Go6976. Figure 4 shows the morphological changes of the HEPCs in the presence of PMA.

As described above, human CD93 has a molecular weight of about 90-100 kDa and is a heavily *O*-glycosylated type I transmembrane protein[1,2] that is mainly expressed on monocytes, granulocytes (neutrophils), endothelial cells, and $CD34^+$ hematopoietic stem cells)[3-6,12]. Furthermore, we also found strong expression of CD93 on the HEPCs in this study. Thus, CD93 may be regarded as a very unique and important molecule in the regulation of various human immune responses.

sCD93 has also been detected in the human serum/plasma of normal adults. Furthermore, cell surface CD93 is reportedly shed from activated human myeloid cells (monocytes and neutrophils) in the form of sCD93 under stimulation by some inflammatory mediators[10,12]. In terms of the biological functions, it has been reported that sCD93 induces differentiation of monocytes and enhances the immunological actions mediated by toll-like receptor (TLR) in various immune responses[14]. In clinical studies, enhanced sCD93 shedding/release has been demonstrated *in vivo* under inflammatory conditions in humans and mice[15,16]; in particular, the sCD93 concentration has been shown to be increased in the synovial fluid/serum of patients with some autoimmune diseases[17]. In this study, as PMA strongly enhanced the secretion of sCD93 from the HEPCs, which was completely suppressed by the PKC inhibitor Go6976. sCD93 secretion from HEPCs is tightly controlled by some phosphorylated proteins through intracellular signal transduction mediated by cPKC. In fact, we reported that CD93 expression on the cell surfaces of cell lines, such as human monocytes (U937), natural killer cells (KHYG-1) and human endothelial cells is strongly enhanced by PMA, and that the CD93 expression is controlled by cPKC at the intracellular signal transduction level[2,11].

Finally, we investigated the effect of CyE, a compound that causes depolymerization of actin filaments, on the secretion of sCD93 from the HEPCs after 6 hrs, 10 hrs and 24 hrs of treatment. Interestingly, marked sCD93 secretion was detected in the culture supernatants of the HEPCs after CyE treatment at 6 hrs (9.6 ng/mL, on average), 10 hrs (12.4 ng/mL, on average), and 24 hrs (19.4 ng/mL, on average). Thus, CyE also significantly ($P < 0.01$) enhanced the secretion of sCD93 by HEPCs as compared to that by the control HEPCs (not treated with CyE). (Fig. 3). In addition, as shown in Figure 4, CyE treatment induces the typical round shape formation of HEPCs via causing depolymerization of actin filaments (Fig. 4).

It has been reported that in general, cytochalasins inhibit cell functions such as chemotaxis, cytokinesis, phagocytosis, and receptor cap formation by causing depolymerization of actin filaments[18]. On the other hand, cytochalasins have also been found to enhance both antigen- and mitogen-induced proliferation of human T cells. For example, when added to T cells, low concentrations of cytochalasins dramatically enhance DNA synthesis as mitogens, resulting in increases of the cyclic AMP (cAMP) and intracellular Ca^{2+} concentrations and phosphorylation of some kinases such as protein kinase A (PKA), protein kinase C (PKC), and protein tyrosine kinase (PTK)[19]. We reported previously that CyE strongly induced low-affinity Fc ε receptor II (CD23) expression on the human monocyte-like cell line, U937[20] and expression of the intercellular adhesion molecule-1(ICAM-1) (CD54) on the HeLa epithelial cell line[21]. These findings indicate that depolymerization of microfilament systems is closely involved in various immunoregulatory mechanisms. Thus, we considered that the enhanced secretion of sCD93 following treatment with CyE may be closely associated with the depolymerization of the microfilament systems induced by the compound.

In conclusion, we found, for the first time, that PMA and CyE treatment strongly induce the secretion of sCD93 from HEPCs. Further analyses are necessary for understanding the detailed intracellular mechanism(s) of sCD93 secretion from HEPCs at the molecular level.

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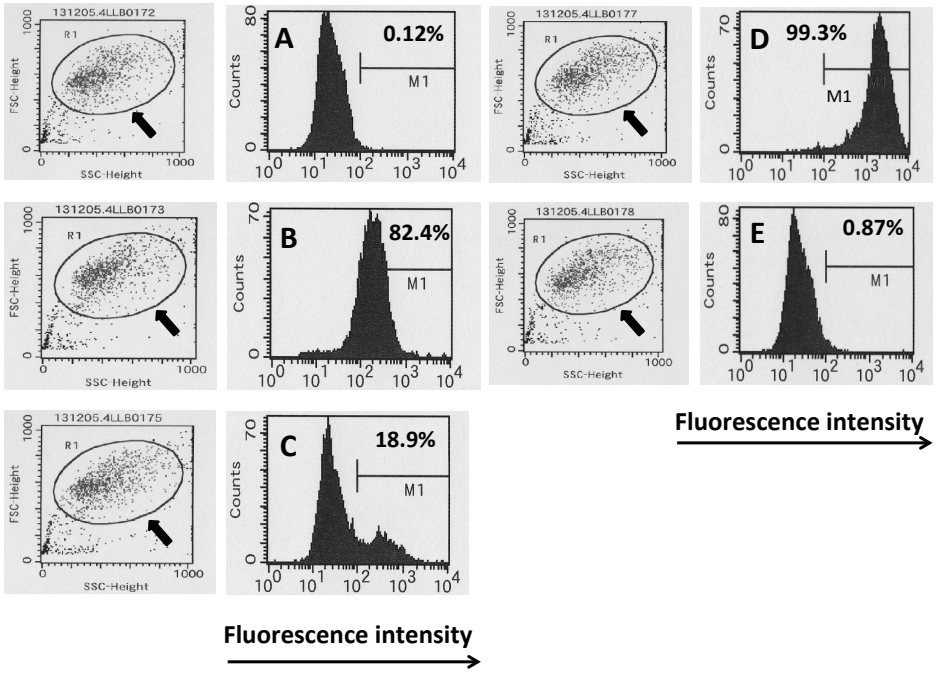

Figure 1. Expression patterns of CD93, CD34, CD146 and CD49d on the HEPCs. The HEPCs were incubated with PE-conjugated CD93 mAb (mNI-11) (panel B), FITC-conjugated CD34 mAb (My10) (panel C), FITC-conjugated CD146 mAb (541-10B2) (panel D) or PE-conjugated CD49d mAb (BU49) (panel E) for 40 min at room temperature. The percentages of positively stained cells for each of the mAbs were analyzed using a single-color flow-cytometric system (See Materials and Methods for details). Percentages are expressed in each panel. Negative control (NC) (panel A) was incubated with mixed PE-conjugated and FITC-conjugated isotype-matched normal mouse IgG1. The arrows within the each panel indicate the analyzed population. The analysis was repeated in triplicate per sample. The experiment was repeated three times.

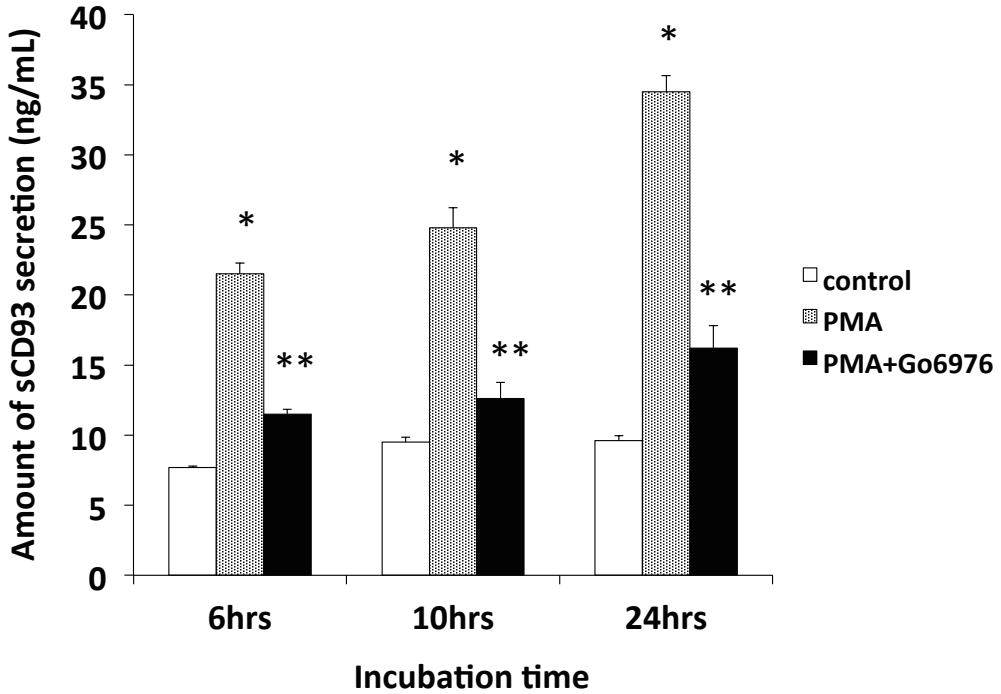

Figure 2. Detection of sCD93 secretion from HEPCs treated or not treated with PMA (100 ng/mL) in the presence or absence of an optimal concentration of a PKC inhibitor (Go6976). The amounts of sCD93 in the culture supernatants of the HEPCs after 6 hrs, 10 hrs and 24 hrs were detected using an EIA system (See Materials and Methods for details). Data are expressed as mean ± SD. The analysis was repeated in triplicate per sample. The experiment was repeated four times. *P < 0.01 (PMA vs. Control). ** P < 0.01 (PMA vs. PMA+Go6976).

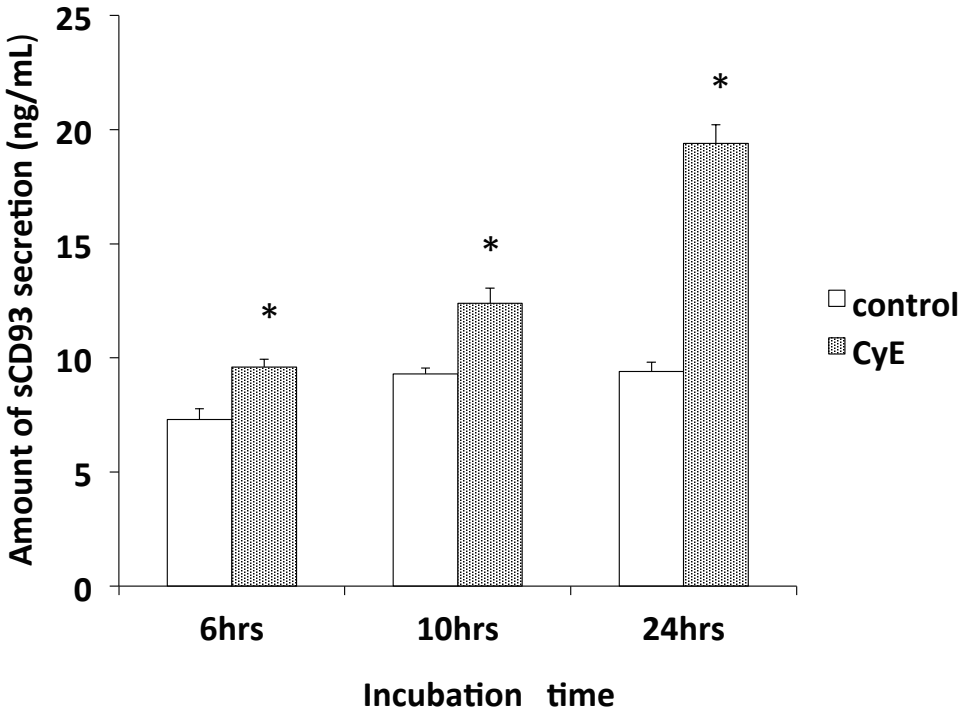

Figure 3. Detection of sCD93 secretion from HEPCs treated or not treated with CyE (100 ng/mL). The amounts of sCD93 in the culture supernatants of the HEPCs after 6 hrs, 10 hrs and 24 hrs were detected using an EIA system (See Materials and Methods for details). Data are expressed as mean ± SD. The analysis was repeated in triplicate per sample. The experiment was repeated three times. *P < 0.01 (CyE vs. Control).

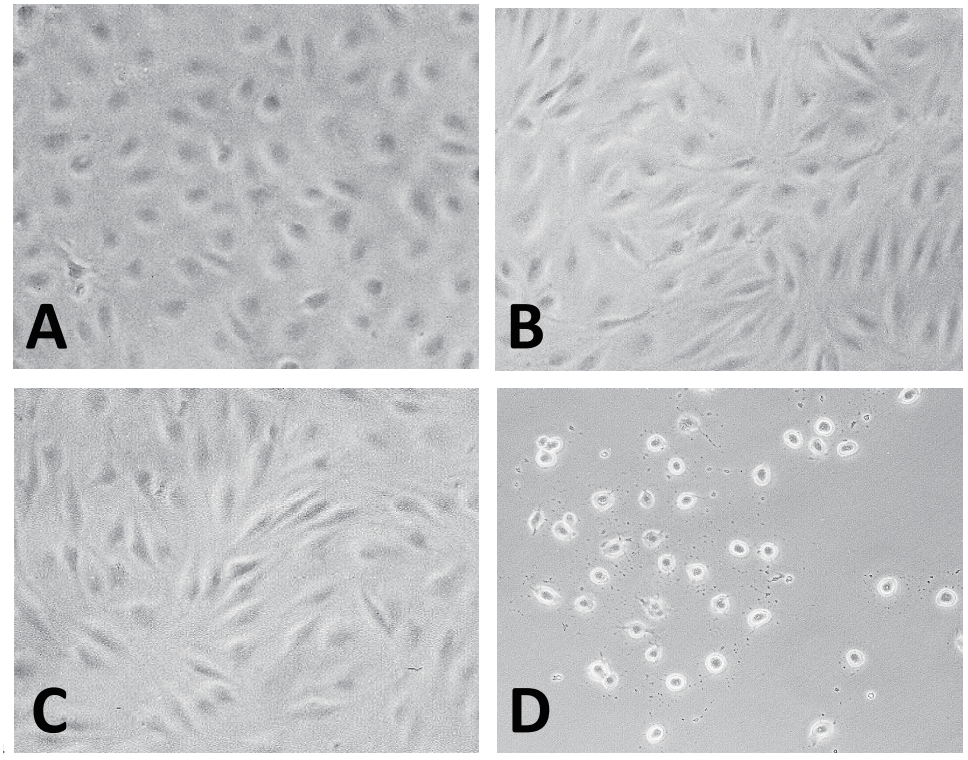

Figure 4. Morphological changes of the HEPCs treated or not treated with PMA or CyE) after 24 hrs (see Materials and Methods for details). The morphological changes of the HEPCs were observed by phase-contrast microscopy (100x). A: control (treated without substance), B: treated with PMA (100 ng/mL), C: treated with PMA (200 ng/mL), D: treated with CyE (100 ng/mL).

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血管内皮前駆細胞から分泌される可溶性 CD93 分子

池脇　信直　　園田　徹 *　　土井　宏太郎 **　　大野　英治 ***

九州保健福祉大学薬学部動物生命薬科学科臨床免疫学研究室　〒 882-8508　宮崎県延岡市吉野町 1714-1
* 九州保健福祉大学保健科学部作業療法学科　〒 882-8508　宮崎県延岡市吉野町 1714-1
** 宮崎県立延岡病院産婦人科　〒 882-0835　宮崎県延岡市新小路 2-1-10
*** 九州保健福祉大学 QOL 研究機構　〒 882-8508　宮崎県延岡市吉野町 1714-1

要　旨

ヒト血管内皮前駆細胞（Human endothelial progenitor cells :HEPCs）から分泌される可溶性 CD93（sCD93）分子の動態を自主開発した抗ヒト CD93 抗体（mNI-11）を用いて解析した。HEPCs は低濃度であるが、sCD93 分子を培養液中に自然分泌していることがわかった。HEPCs を PKC 活性剤である PMA で処理したところ、sCD93 分子の分泌は有意に増強した（$P < 0.01$）。また、PMA 処理の初期段階から sCD93 分泌の増強は認められた。次に、PMA 処理 HEPCs に PKC 活性阻害剤である Go6976 を添加したところ、sCD93 分子の分泌は有意に抑制された（$P < 0.01$）。一方、HEPCs をアクチンフィラメントの脱重合剤（サイトカラシン E）で処理したところ、sCD93 分子の分泌が有意に増強した（$P < 0.01$）。HEPCs から分泌される sCD93 分子は、PKC によるリン酸化とアクチンフィラメントの脱重合が密接に関与していることがわかった。。

キーワード：サイトカラシン E、ヒト血管内皮前駆細胞、ホルボールミリステートアセテート、
可溶性 CD93 分子