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Original Article

Inhibitory Effects of CD99-derived Peptide CD99CRIII3 on the Extravasation of Monocytes and Inflammatory Reactions in Contact Dermatitis Mouse Model

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Abstract : Leucocyte extravasation has been known to play an important role in inflammatory reactions including contact dermatitis. Previous studies suggested that CD99 regulates β1 integrin activity and may be a novel therapeutic target molecule for inflammatory diseases. In this study, the effects of CD99-derived peptide, CD99CRIII3, on inflammatory reactions in contact dermatitis mouse model were investigated. CD99CRIII3 decreased β1-integrin activity in human monocytic U937 cells. CD99CRIII3 inhibited the adhesion of U937 monocytes to human umbilical vein endothelial cells and their extravasation through human umbilical vein endothelial cells. CD99CRIII3 reduced inflammation in the phorbol myristate acetate-induced contact dermatitis mice in a dose-dependent manner. These results indicate that CD99CRIII3 suppresses the extravasation of monocytes and inflammatory reactions in the animal model of the contact dermatitis, suggesting that CD99CRIII3 could be a new drug candidate against inflammatory skin diseases.

Keywords : CD99-derived peptide, Contact dermatitis, CD99, Inflammation

Introduction

Dermatitis is a polymorphic inflammation of the skin. There are multiple forms of dermatitis, the most common being atopic dermatitis and contact hypersensitivity [1]. The evolution of contact dermatitis skin lesions is orchestrated by the local tissue expression of proinflammatory cytokines and chemokines. Cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) from resident cells (keratinocytes, mast cells, and dendritic cells) bind to receptors on vascular endothelium, activating cellular signaling including the nuclear factor kappa light chain enhancer of activated B cells (NF-kB) pathway and inducing expression of vascular endothelial cell adhesion molecules (VCAM) [2,3]. These events initiate the process of tethering, activation, and adhesion to the endothelium followed by extravasation of inflammatory cells. Once the inflammatory cells have infiltrated into the tissue, they respond to chemotactic gradients established by chemoattractant cytokines and chemokines, which emanate from sites of injury or infection [3,4]. These molecules play a central role in defining the nature of the inflammatory infiltration in contact dermatitis [5].

CD99 is an endothelial membrane protein that participates in the transmigration of monocytes through human umbilical vein endothelial cells (HUVECs) *in vitro* [6-12].

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CD99 is expressed on most leukocytes in which its function is not well understood. CD99 has been described as a costimulatory molecule on T cells and a target for antibody-induced integrin-independent aggregation of thymocytes, β2-integrin–dependent aggregation of a B lymphoblastoid cell line, α4β1-integrin–mediated adhesion of T cells to endothelial vascular cell-adhesion molecule 1 (VCAM-1), and apoptosis [13-17]. Recently, we reported that CD99CRIII3, a CD99-derived tripeptide, inhibits fibronectin–mediated β1-integrin activation, suggesting that it can suppress β1-integrin–mediated inflammatory reactions [19-23].

In this study, we investigated the effects of CD99CRIII3 on transendothelial migration of monocytes and in a contact dermatitis mouse model. It inhibited not only adhesion of monocytes to endothelial cells but also *in vitro* transendothelial migration of monocytes. In addition, it reduced the inflammation in the Phorbol myristate acetate (PMA)-induced contact dermatitis in a dose-dependent manner. These studies suggest that CD99CRIII3 can be developed as a therapeutic agent for chronic inflammatory diseases such as contact dermatitis.

Materials and Methods

1. Reagents

Culture reagents were purchased from Invitrogen (Carlsbad CA, USA). Mouse anti-human β1 integrin was obtained from Millipore (Burlington MA, USA) and horseradish peroxidase (HRP)–Immunoglobulin G (IgG)-conjugated anti-mouse IgG antibody was purchased from DiNonA, Inc. (Seoul, Korea). Transwells (8.0 μm pore size) were obtained from Corning. (NY, USA).

2. Cell culture

MCF-7 human breast cancer cell line and U937 Human monocytic cell line was cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL Streptomycin (Gibco, NY, USA), and 1 mM sodium pyruvate. bEnd.3 mouse endothelial cell was cultured in Dulbecco's modified Eagle's medium (DMEM) supplement with 1.5 g sodium carbohydrate, 10% v/v fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL Streptomycin. WEHI 274.1 mouse monocytic cell line was cultured in DMEM supplement with 3.7 g sodium carbohydrate, 10% v/v fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL Streptomycin. Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% CO_2.

3. Synthesis of peptides

The polypeptide CD99CRIII3 containing sequences derived from CD99 were synthesized with an automatic peptide synthesizer (PeptrEx-R48, Peptron, Daejeon, Korea) using a 9-fluorenylmethoxycarbonyl (FMOC) solid-phase method. The synthesized polypeptides were purified and analyzed by reverse-phase high-performance liquid chromatography (reverse-phase HPLC) (Prominence LC-20AB, Shimadzu, Japan) using a C18 analytical Reversed Phase (RP) HPLC column (Shiseido Capcell Pak, Tokyo, Japan), and isolated using a mass spectrometer (HP 1100 Series LC/MSD, Hewlett-Packard, Roseville, USA.).

4. Western blot analysis

U937 cells were resuspended at a density of 1×10^6 cells/mL in serum free medium in a 1.5 mL tube and peptides treated for 1 hour. Cells were lysed in nonidet P40 (NP-40) lysis buffer. Cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was subsequently transferred to a nitrocellulose membrane.

5. Cell-cell binding assay

bEnd3 cells were plated onto filter inserts of a 96-well Transwell plate in growth medium and grown to confluence for 24 h in 5% CO_2 at 37°C. Then, mouse monocyte WEHI274.1 cell suspension, pretreated with or without CD99CRIII3, was added in the upper compartment of the wells. After 6 h, the cells were harvested and quantitated by using a hematocytometer under an inverted microscope. Assays were carried out in triplicates.

6. Transendothelial migration assay

bEnd3 cells were plated onto filter inserts (24-well plate, pore size 8.0 μm) in growth medium, and grown to conflu-

ence for 24 h in 5% CO_2 at 37°C. Later, mouse monocyte WEHI274.1 cell suspension, pretreated with or without CD99CRIII3, was added to the upper compartment of the chamber. After 6 h, the cells in the lower compartment of the chamber were harvested and quantitated by using hematocytometer under an inverted microscope. Assays were carried out in triplicates.

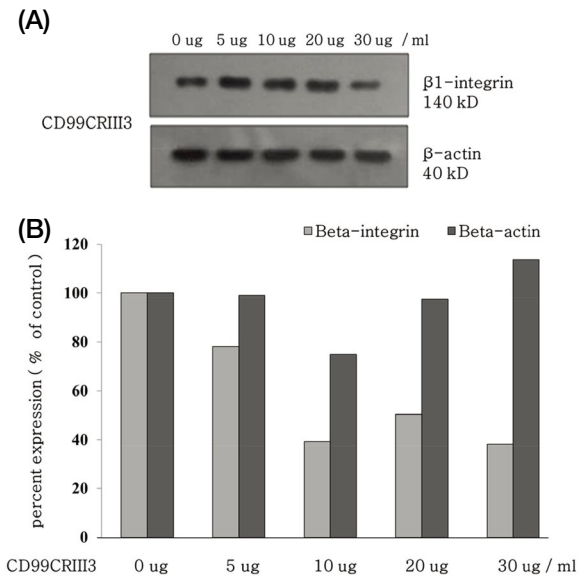

Fig. 1. CD99CRIII3 inhibit β1-integrin activity in a dose dependent manner. U937 cells were treated with various amount of CD99CRIII3 and then subjected to western blot analysis (A). Relative activity levels of β1-integrin in (A) are shown as relative intensity of bands by measuring with a densitometer (B).

7. Animals

Male ICR mice (6-weeks old) were purchased from the Orientbio Experimental Animal Center (Seoul, Korea) and were housed in the animal facilities at the College of Medicine in Kangwon National University. Four mice were housed per cage fitted with a laminar airflow cabinet. The mice were kept at 22 ± 11°C and a relative humidity of 55 ± 10% in a 12-hour light-dark cycle. This study was performed in accordance to the Institutional guidelines.

8. Treatment of animals

Phorbol myristate acetate (PMA) was used as an inducer of skin inflammation. Twenty microliters of PMA (250 μM) were painted to dorsal surface of right ears to induce skin inflammation and 20 μL of acetone : olive oil (4 : 1) was painted to dorsal surface of left ears to control. The CD99CRIII3 melts in PBS and medicates. The processing of the CD99CRIII3 does the tail vein injection before the PMA coating [23-26].

9. Assessment of the effects of PMA treatment

After 4∼6 h, the mice were challenged with intravenous injection of PBS containing 4% Evans blue solution. One hour later, the mice were killed followed by the removal

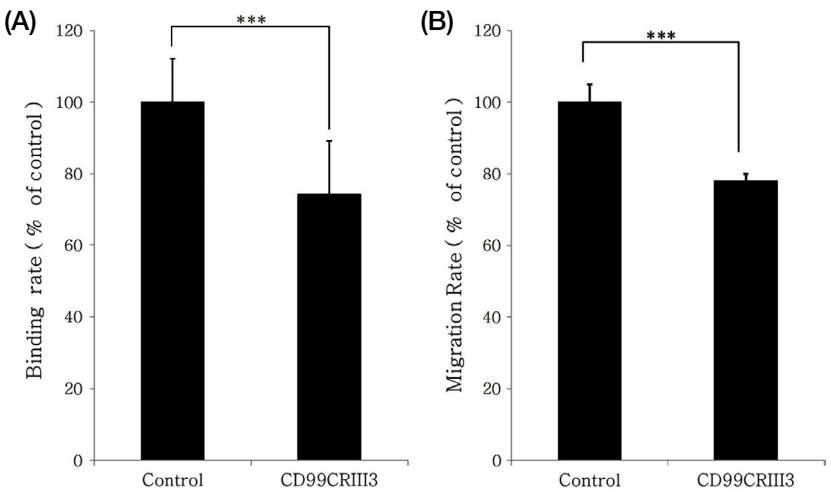

Fig. 2. Effects of CD99CRIII3 on cell-cell adhesion and transendothelial migration of monocytes. WEHI 274.1 monocytes were treated with CD99CRIII3 or control peptide and subjected to cell-cell binding assay (A) and were also subjected to transendothelial migration (B). Results are the mean ± S.E. of triplicate assays: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

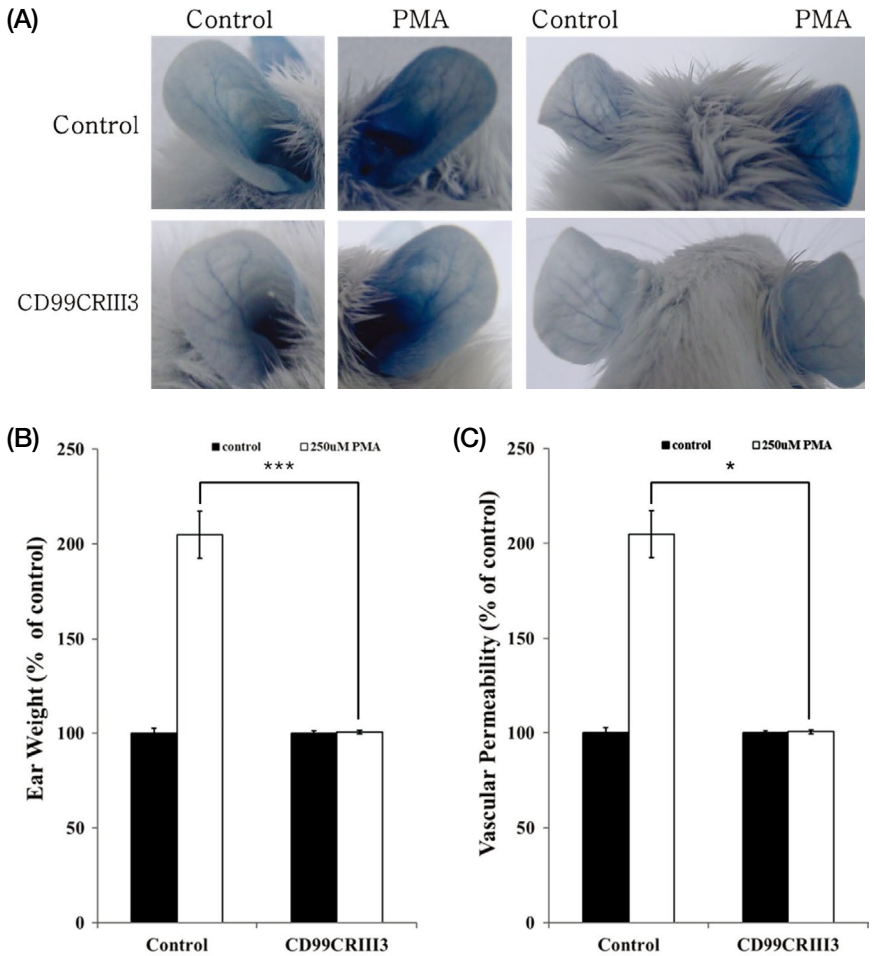

Fig. 3. CD99CRIII3 suppresses inflammatory reaction in a murine model of acute contact dermatitis. (A) After PMA-mediated induction of contact dermatitis in mice, CD99CRIII3 or control peptide were injected into tail vein of mice. After 6 h, vascular permeability was measured by injection of Evans Blue dye and observed with the naked eye. (B) Ears were removed from the mice treated as described in Figure 3(A). After soaking in formamide solution overnight, the amount of Evan Blue leakage from the vessels was measured by a spectrophotometer. (C) Ear pieces were removed from mice treated as described in Figure 3(A), and the ear pieces weighed. Results are the mean ± S.E. of triplicate cultures: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

of the ear for measurement of the amount of extravasated dye. The dye was eluted from the ear in 1 mL of formamide at 37°C and absorbance was measured at 620 nm [23-26].

Results

1. CD99CRIII3 inactivates β1-integrin in U937 cells

To investigate whether CD99-derived tripeptide CD99CRIII3 can inactivate β1-integrin, U937 cells were treated with various amounts of CD99CRIII3 and then were subjected to western blot analysis using anti-β1-integrin monoclonal antibody that specifically recognizes the active form of β1-integrin. As shown (Fig. 1), CD99CRIII3 inactivates β1-integrin in a dose-dependent manner.

2. CD99CRIII3 attenuates the adhesion of monocytes to endothelial cells

As CD99CRIII3 inactivates β1-integrin, we hypothesized that it can decrease the adhesion of monocytes to endothelial cells by inhibiting very late antigen 4 (VLA-4), α4β1-integrin activity. The treatment of mouse monocyte WEHI 274.1 cells with CD99CRIII3 significantly decreased their adhesion to endothelial cells (Fig. 2A). This result suggests that CD99CRIII3 may inhibit extravasation of monocytes through suppression of their adhesion

to endothelial cells. To examine whether CD99CRIII3 can inhibit transendothelial migration of monocytes, we performed monocyte transendothelial migration analysis using CD99CRIII3 under static conditions. CD99CRIII3 treatment of WEHI274.1 cells led to a decrease in their transendothelial migration (Fig. 2B). Taken together, these results demonstrate that CD99CRIII3 ameliorates transendothelial migration of monocytes.

3. CD99CRIII3 ameliorates inflammatory reactions in a murine model of PMA-induced acute contact dermatitis in a dose dependent manner

To examine the effects of CD99CRIII3 on contact dermatitis, one of representative inflammatory diseases, we investigated its effect on the pathological progression of contact dermatitis in mice. To measure vascular permeability, 6 h after the induction of contact dermatitis, mice were intravenously injected with Evans blue dye and were observed for vascular permeability in the ears by the naked eye (Fig. 3A). In addition, the amount of Evans blue dye leakage into the ear dermis was measured by a spectrophotometer after soaking the ears in PBS (Fig. 3B). Compared to control, mice treated with CD99CRIII3 showed a decrease in the dye leakage into the ear dermis, suggesting that CD99CRIII3 significantly suppresses vascular permeability. In addition, the ear pieces were weighed (Fig. 3C). It was observed that CD99CRIII3 decreased the ear weights. Together, our results suggest that CD99CRIII3 suppresses contact dermatitis through its anti- inflammatory activities.

Discussion

Our results indicate that CD99-derived tripeptide functions as a novel suppressor of β1 integrin activation and prevents progression of contact dermatitis in mice. This study is consistent with our previous study showing that CD99-derived agonistic ligands function as novel suppressors of fibronectin-mediated β1 integrin activation in human breast carcinoma cells [19].

Several mechanisms may account for the suppressive effect of CD99CRIII3 on the progression of contact dermatitis in mice. Previous studies showed that treatment of monocytes, neutrophils, or endothelial cells with CD99 blocking antibodies suppressed diapedesis of leucocytes and inflammatory reactions in mice. CD99 blocking antibodies might interrupt homophilic interaction of CD99 molecules on the leucocytes and endothelial cells, suggesting that CD99 is involved in the regulation of the final step of emigration of leucocytes, wherein leukocytes migrate through capillary vessels and basement membranes. Moreover, CD99 can inhibit leucocyte extravasation by regulating β1 integrin activity. Our previous studies demonstrated that CD99-derived agonist ligands inhibit fibronectin-induced activation of β1 integrin through the protein kinase A (PKA)/Src homology 2 domain-containing protein tyrosine phosphatase (SHP2)/extracellular signal-regulated kinase (ERK1/2)/Protein Tyrosine Phosphatase, Non-Receptor Type 12 (PTPN12)/focal adhesion kinase (FAK) signaling pathway by specifically interacting with the three conserved motifs in the CD99 extracellular domain [19]. CD99 activation leads to a decrease in cell-extracellular matrix adhesion by suppressing β1 integrin activity. Correspondingly, pretreatment of monocytes with CD99-stimulating peptide, PILRpep3, decreased the adhesion of monocytes to endothelial cells, which could lead to reduction of the whole emigration process of monocytes [27]. Thus, the anti-β1 integrin activity of CD99 activation allow us to explain the effects of CD99-derived tripeptide on progression contact dermatitis in mice [18].

In conclusion, our findings demonstrate that CD99CRIII3 suppresses inflammatory reactions in the animal model of the contact dermatitis, suggesting that CD99 peptidomimetics could be novel therapeutic drug candidates for inflammatory diseases.

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접촉성 피부염 마우스 모델에서 단핵구의 유출 및 염증 반응에 대한 CD99-유래 펩타이드 CD99CRIII3의 억제 효과

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간추림 : 접촉피부염은 피부가 이물질과 접촉함으로써 일어나는 염증반응으로 백혈구 혈관외유출이 매우 중요한 역할을 한다는 것은 잘 알려진 사실이다. 선행연구 결과 CD99가 β1 인테그린 의존적 메커니즘을 통하여 단핵구의 혈관외유출을 조절하며, 따라서 염증 질환에 대한 치료제 개발의 신규 표적 분자일 가능성이 제시되었다. 본 연구에서는 CD99 유래 펩타이드인 CD99CRIII3가 단핵구의 혈관외유출과 접촉피부염 생쥐 모델에서 염증 반응을 억제하는지를 조사하였다. 인간 단핵구 세포주인 U937를 CD99CRIII로 처리할 경우 농도의존적으로 β1 integrin의 활성도가 감소되었으며, 이 세포주의 사람제대정맥내피세포에의 부착과 혈관외유출도 억제되었다. 나아가 CD99CRIII3는 포르볼 미리스테이트 아세테이트 처리에 의해 유발된 생쥐 접촉피부염 모델에서 Evans Blue의 혈관투과와 귀조직 무게를 농도 의존적으로 억제하였다. 이러한 결과들은 CD99CRIII3가 단핵구의 혈관외유출과 접촉피부염 동물 모델에서 일어나는 염증반응을 억제함을 보여준다. 이와 같이, 본 연구는 CD99 유래 펩타이드가 피부 염증질환에 대한 치료제로 개발될 가능성이 있음을 제시한다.

찾아보기 낱말 : CD99-유래 펩타이드, 접촉피부염, CD99, 염증

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