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The activation of CD99 inhibits cell-extracellular matrix adhesion by suppressing β_1 integrin affinity

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CD99 is known to be involved in the regulation of cell-cell adhesion. However, it remains unclear whether CD99 controls cell-extracellular matrix adhesion. In this study, the effects of CD99 activation on cell-extracellular matrix adhesion were investigated. It was found that engagement of CD99 with the stimulating antibody YG32 downregulated the adhesion of MCF-7 cells to fibronectin, laminin and collagen IV in a dose-dependent manner. The CD99 effect on cell-ECM adhesion was inhibited by overexpression of the dominant negative form of CD99 or CD99 siRNA transfection. Treatment of cells with Mn^{2+} or by β_1 integrin-stimulating antibody restored the inhibitory effect of CD99 on cell-ECM adhesion. Cross-linking CD99 inactivated β_1 integrin through conformational change. CD99 activation caused dephosphorylation at Tyr-397 in FAK, which was restored by the β_1 stimulating antibody. Taken together, these results provide the first evidence that CD99 inhibits cell-extracellular matrix adhesion by suppressing β_1 integrin affinity. [BMB reports 2012; 45(3): 159-164]

INTRODUCTION

The regulation of β_1 integrin activity through "inside-out signaling" plays a significant role in a variety of cell behaviors, in both pathological and normal physiology (1, 2). Several receptor molecules have been previously shown to regulate β_1 integrin activity; these include CD47, CD98 and the tetraspan family (TM4SF) of receptors, which includes CD9, CD81 and CD151. It was found that the binding of thrombospondin-1 (TSP 1) or peptides from TSP 1 to CD47 led to activation of β_1 integrin in vascular smooth muscle cells (3). CD98 activation induced not only conformational changes of β_1 integrin but also changes in its surface expression and clustering (4, 5). It has been suggested that CD9 enhances cell motility to fibronectin by activating β_1 integrin via a PI-3 kinase-dependent pathway (6). CD81 and CD151 form a stable complex with β_1 integrin, which potentiates the ligand-binding activity of the integrin through sustaining its activated conformation (7, 8). On the other hand, chemorepulsive signal receptors such as semaphorins and ephrins negatively regulate β_1 integrin; for example, previous study showed that activation of Plexin-B1 by Semaphorin 4D inhibited β_1 integrin-mediated adhesion via inactivating R-Ras (9, 10). It was also shown that EphA2 activation suppressed integrin mediated cell adhesion, spreading and migration by inducing conformational change of β_1 integrin (11).

The human CD99 protein is a 32 kDa type I transmembrane glycoprotein which is broadly expressed on many cell types, but which is abundant on lymphocytes and on several tumors including Ewing's sarcoma (12-14). The CD99 protein has been implicated in various cellular processes, including apoptosis, vesicular protein transport, thymocyte differentiation, and leucocyte diapedesis (15-19). Several lines of evidence indicate that CD99 may be involved in the regulation of cell-cell adhesion as well. For example, cross-linking of CD99 molecules with a monoclonal antibody was shown to induce homotypic aggregation of lymphocytes through up-regulation of LFA-1 ($\alpha_L\beta_2$ integrin) activity and expression (13, 20). In addition, CD99 engagement induced the adhesion of T lymphocytes to VCAM-1-expressing endothelial cell monolayers by stimulating $\alpha_4\beta_1$ (21, 22). These results led us to hypothesize that CD99 could be involved in the regulation of cell-ECM interaction as well. This study provides evidence that the activation of CD99 drastically reduces the adhesion of MCF-7 cells to fibronectin, laminin, and collagen IV by attenuating β_1 integrin affinity.

RESULTS

CD99 engagement inhibits β_1 integrin-mediated cell-ECM adhesion

To investigate whether CD99 activation leads to any change in adhesion of MCF-7 cells to ECM, we treated MCF-7 cells with

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CD99 activating mAb YG32 (23) and anti- mouse IgG as a secondary Ab for one hour and then allowed adherence to fibronectin, laminin, collagen V or poly-L-lysine (PLL) coated plates in the absence of serum. As shown in Fig. 1A, CD99 is abundantly expressed in MCF-7 cells. Cross-linking CD99 decreased cell adhesion to fibronectin, laminin, and collagen IV (Fig. 1B). In contrast, adhesion to poly-L-lysine, which does not engage integrins, was not affected by CD99 activation. When MCF-7 cells were incubated in the presence of increasing concentrations of YG32, a dose-dependent decrease in adhesion was seen (Fig. 1C). When the MCF-7 cells were treated with mouse IgG1 and anti-mouse IgG, the level of cell adhesion to ECM proteins was similar to that of untreated MCF-7 cells.

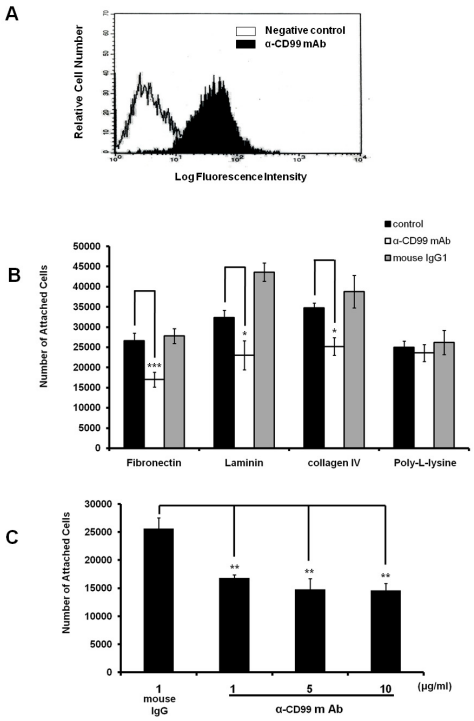

Fig. 1. Depiction of how cross-linking of CD99 inhibits adhesion of MCF-7 cells to extracellular matrix. (A) FACS analysis was used to examine the cell surface expression of CD99 on MCF-7 cells. Binding of the anti-human CD99 monoclonal antibody against CD99 molecules expressed on the cell surface was induced and the cells were then stained with an FITC-conjugated anti-mouse IgG antibody. (B) MCF-7 cells were treated with the anti-CD99 monoclonal antibody YG32 and goat anti-mouse IgG (whole molecule). 1 hour after incubation, the number of MCF-7 cells attached to fibronectin, laminin, collagen IV and poly-L-lysine were determined as described in the "Materials and Methods" section. (C) MCF-7 cells were incubated with increasing amounts of the anti-CD99 monoclonal antibody and then an adhesion assay was performed. Results were replicated in three independent experiments. A significant difference from the control is shown by asterisks as follows: *P < 0.05; **P < 0.01, ***P < 0.001. Lines indicate additional statistical comparisons.

Loss of CD99 function diminishes the inhibitory effect of CD99 on cell adhesion

To confirm whether or not the effect of CD99 engagement on the adhesion of MCF-7 cells to ECM is mediated by CD99 molecules on the cells, we obtained a dominant-negative (DN) CD99-EGFP fusion construct. Dominant negative CD99-EGFP chimeric molecules contain the extracellular and transmembrane domain of CD99 fused to EGFP. After MCF-7 cells were transfected with the DN CD99-EGFP fusion construct, stably transfected MCF-7 cells showing high level of CD99 were selected (Fig. 2A). We confirmed the expression of DN CD99-EGFP using Western blot analysis and confocal microscopy (Fig. 2B and C). Fig. 2D shows that overexpression of dominant negative CD99 reversed the inhibitory effect on CD99 on the cell-ECM adhesion.

To further examine whether CD99 activation inhibits cell-ECM adhesion, we transiently transfected MCF-7 cells with siRNA for the CD99 gene and then treated them with anti-CD99 mAb YG32. Western blot analysis revealed a decrease in CD99 expression in the cells that had been transiently transfected with CD99 siRNA, compared to the control cells transfected with control siRNA (Fig. 2E). As shown in Fig. 2F, treatment with CD99 siRNAs inhibited the CD99-mediated suppression of adhesion of MCF-7 cells to fibronectin. Taken together, these results indicate that antibody-induced CD99 signaling down-regulates adhesion of MCF-7 cells to ECM molecules through CD99.

Cross-linking CD99 induces conformational change of β_1 integrin

To examine whether inhibition of cell adhesion by CD99 signaling derives from conformational changes in integrin, we treated MCF-7 cells with Mn^{2+} that converts integrin into an active conformation (24), before stimulating cells with CD99 mAb. Fig. 3A shows that the addition of Mn^{2+} reversed the inhibitory effects of CD99 in a concentration-dependent manner. Since the common ligand for fibronectin, laminin, and collagen IV is β_1 integrin, we investigated whether or not treatment of cells with the β_1 integrin-stimulating antibody B44 reverses CD99's effect on the adhesion of cells to fibronectin (24). As shown in Fig. 3B, activation of β_1 integrin diminished the inhibitory effect of anti-CD99 mAb on cell-ECM adhesion in a dose-dependent manner. In fact, Fig. 3C shows that the CD99 monoclonal antibody YG32 converted β_1 integrins to an inactive conformation in a dose and temperature-dependent manner (25). These results indicate that CD99 activation converted β_1 integrins into an inactive conformation, thereby decreasing cell binding to extracellular matrix.

Next, we determined whether CD99-induced inactivation of β_1 integrin leads to a dephosphorylation of focal adhesion kinase (FAK), which is a key regulatory molecule in the downstream signaling events that occur after integrin activation (26). The effect of CD99 activation on the phosphorylation status of FAK in MCF-7 cell attached to the matrix was examined by

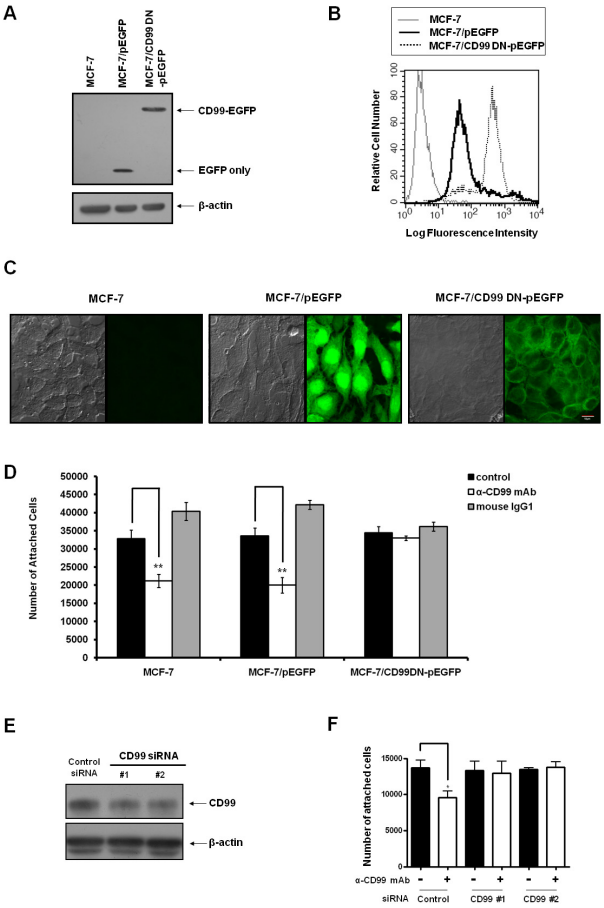

Fig. 2. The overexpression of the dominant negative form of CD99 or the transfection of the CD99 siRNA restored CD99 mAb-mediated reduction of adhesion of MCF-7 cells to fibronectin. (A) MCF-7 cells were transfected with a mock expression vector pEGFP-N2 alone or with a pEGFP-N2 vector encoding dominant-negative CD99. The expression of GFP in the transfectants and parental MCF-7 cells were examined using monoclonal antibodies specific for GFP. (B) Cell surface expression levels of CD99 in the transfectants and parental MCF-7 cells were determined by flow cytometry using an anti-human CD99 monoclonal antibody and an FITC-conjugated anti-mouse IgG antibody. Fluorescence intensity is plotted on the abscissa and cell number on the ordinate. (C) Expression of EGFP protein in the transfectants was analyzed under a confocal microscope. (D) Adhesion analysis with dominant negative CD99 transfectants was carried out as described in the "Materials and Methods" section. Parental MCF-7 cells and mock transfectants (MCF-7/pEGFP) were used as control groups. (E) After transfection of CD99 siRNAs into MCF-7 cells, CD99 expression levels were determined using monoclonal antibodies specific for CD99. (F) The MCF-7 cells trasfected with CD99 siRNAs were applied to adhesion analysis as was described above. A significant difference from the control is shown by asterisks as follows: *P < 0.05; **P < 0.01. Lines indicate additional statistical comparisons.

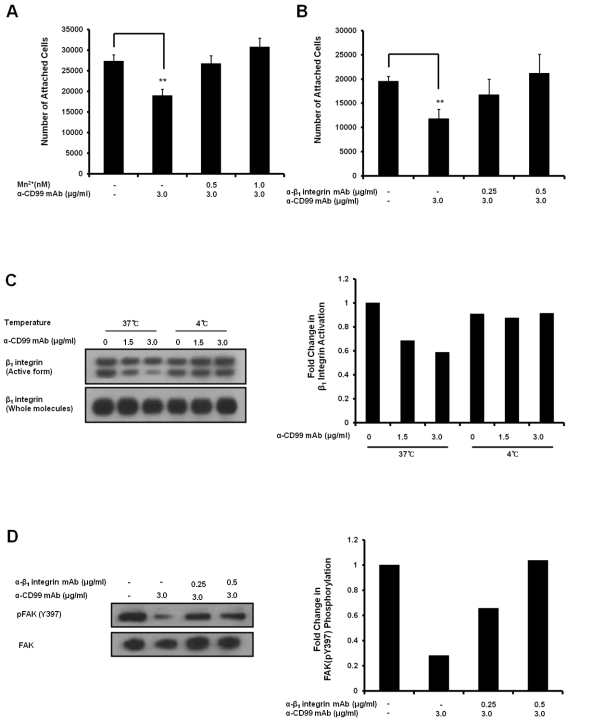

Fig. 3. CD99 activation converts β_1 integrin into an inactive conformation and dephosphorylates FAK. MCF-7 cells were incubated with increasing amounts of Mn^{2+} (A) or with the anti-β_1 integrin stimulating antibody B44 (B) in the presence of the mouse anti-human CD99 antibody and then their effects on the inhibitory function of CD99 in cell-ECM adhesion were examined. (C) After MCF-7 cells were treated with a mouse anti-human CD99 monoclonal antibody at 4°C or 37°C for 1 h, conformational change of β_1 integrin was examined with immunoblot anaylsis using a monoclonal antibody (B44) specific for the active form of β_1 integrin as well as a monoclonal antibody (JB1A) against the total β_1 integrin. The graph represents the densitometric values of immunoblotting, which show the ratio between the active form of β_1 integrin and the total β_1 integrin. (D) MCF-7 cells were incubated with increasing amounts of the anti-β_1 integrin stimulating antibody B44 with an anti-CD99 monoclonal antibody (3 μg/ml) and then phosphrylation of FAK was determined by immunoblotting using monoclonal antibodies against phosphorylated FAK (pY397) or a polyclonal antibody against total FAK. Quantitation of the phosphorylated FAK was carried out as described in (C). A significant difference from the control is shown by an asterisk as follows: **P < 0.01. Lines indicate additional statistical comparisons.

immunoblotting. Fig. 3D shows CD99 induced FAK dephosphorylation at tyrosine 397 in MCF-7 cells attached to the matrix, which was restored by β_1 stimulating antibody B44. These results suggest that FAK may be involved in CD99-mediated suppression of β_1 integrin activity.

DISCUSSION

Our study has shown a new function of CD99: as an important

regulator of cell-ECM adhesion. Specifically, we have provided the first evidence to show that CD99 activation converted β_1 integrin to an inactive conformation, thereby inhibiting cell-ECM adhesion. CD99 YG32 mAb decreased the adhesion of human breast carcinoma MCF-7 cells to fibronectin, laminin and collagen IV in dose-dependent manner, which was restored by overexpression of dominant-negative CD99 or CD99 siRNA transfection. Interestingly, the CD99 effect on the adhesion of cells to the fibronectin was reversed by the treatment of cells with Mn^{2+} or by stimulating antibody B44, which is known to activate β_1 integrins through conformational change (24). In addition, our immunoblotting experiments clearly showed that CD99 activation led to a significant reduction in the expression level of the active form of β_1 integrins, but not in the expression level of the total β_1 integrins. This reduction was also not observed when MCF-7 cells were treated with anti-CD99 mAb at 4°C (25). Furthermore, FACS analysis did not show that there was any change in the expression of β_1 integrin in the MCF-7 cells after CD99 mAb treatment (data not shown). Taken together, these results suggest that CD99 negatively regulates the conformation of β_1 integrins rather than their internalization or lysosomal degradation.

Previous study has, by contrast, shown that CD99 engagement leads to the enhancement of $\alpha4\beta_1$ integrin activity in peripheral T cells (21). When human T cells such as Jurkat cells, CEM-T, and peripheral memory T cells were stimulated with the CD99 O662 mAb, up-regulation of their firm adhesion and cell spreading on the TNFα-treated endothelial cells was observed under physiological shear stress. In addition, stimulation of CD99 isoform transfectants with CD99 O662 mAb induced T-cell arrest on human recombinant VCAM-1 monolayers, suggesting that CD99 activation upregulates $\alpha_4\beta_1$ integrin activity (22). This conflict between our results and those of earlier studies may be due to differences in the antibodies used in the experiments. Specifically, CD99 O662 mAb may upregulate the expression level or activity of α_4 integrins, whereas CD99 YG32 mAb inactivates β_1 integrin activity. Under our experimental conditions, CD99 activation by natural ligands such as PILR and CD99 itself as well as by monoclonal antibodies led to a reduction in cell-ECM adhesion and cell spreading on ECM, strongly suggesting that CD99 is a negative regulator of β_1 integrin (data not shown).

Suppression of β_1 integrin activity plays a significant role in the regulation of a variety of cellular processes including cell survival, proliferation, adhesion, and migration. For example, it was shown that overexpression of CD82 inactivated β_1 integrin led to significantly reduced cell motility (27) and that Sema3A inhibited neoangiogenesis in tumors by inhibiting β_1 integrin activation (28). Other study showed that EphA4 attenuates β_1 integrin activation, which destabilizes dendritic spines in the nervous system (29, 30). EphA2 activation negatively regulated β_1 integrin and FAK (11). Likewise, stimulation of CD99 led to not only inactivation of β_1 integrin but to dephosphorylation of FAK, both of which were restored by β_1 integrin stimulating antibodies. Thus, CD99 may play an important role in the regulation of cell-cell adhesion and in cell-ECM adhesion in both normal and abnormal human physiology. Interestingly, CD99 has been found to regulate the transendothelial migration of neutrophil and monocytes (15, 31-35). However, the molecular mechanisms underlying CD99-mediated diapedesis of leucocytes remain largely unknown. Our study may provide some information about the role of CD99 in transendothelial migration of leucocytes in the inflammatory reactions.

In summary, our study shows that CD99 has a function in the regulation of cell-extracellular matrix adhesion, which provides a basis for understanding how β_1 integrin regulators act. Future studies investigating CD99 ligands and signaling pathways involved in β_1 integrin inactivation may ultimately lead to the creation of new strategies for controlling inflammatory diseases.

MATERIALS AND METHODS

Materials

Fibronetin, laminin and all culture reagents were purchased from Invitrogen (Carlsbad, CA). The collagen IV, poly-L-lysine and $MnCl_2$ were from the Sigma-Aldrich Co. (St. Louis, MO). The Immune-Blot PVDF membrane for protein blotting was purchased from Bio-Rad laboratories (Hercules, CA). The antibody detection kit (West-Zol plus) was obtained from iNtRON Biotechnology, Inc. (Seongnam, S. Korea). Anti-human CD99 mAb (YG32), goat anti-mouse IgG (whole molecule), FITC-conjugated anti-mouse IgG antibody and the HRP-conjugated anti-mouse IgG antibody were both purchased from DiNonA (Seoul, S. Korea). The mouse anti-human integrin β_1 monoclonal antibody (JB1A), mouse anti-integrin β_1 preservative-free monoclonal antibody (B44), and HRP-conjugated goat anti-rabbit IgG antibody were purchased from Chemicon (Temecula, CA). The mouse anti-GFP mAb and anti-FAK rabbit polyclonal antibody (C-20) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The purified mouse anti-human FAK (pY397) monoclonal antibody (14/FAK Y397) was from BD Biosciences (San Jose, CA). Anti-human β-actin mAb, mouse IgG purified immunoglobulin, and anti-human IgG (Fc specific) antibodies were from Sigma-Aldrich Co. (St. Louis, MO).

Cell culture

Human breast carcinoma cells (MCF-7) were obtained from the American Type Culture Collection (ATCC) and were cultured as described previously (36).

DNA constructs and transfection

The dominant negative CD99 DNA fragment, which includes only extracellular and transmembrane regions, was obtained by PCR using 10 pmol of sense primer (5′-CGGAATTCCGG CGCACCATGGCCCGCGG-3′) and antisense primer (5′-GGA

ATTCAGCAATGAAGCTAGAGATGGC-3′). To obtain the dominant-negative CD99-EGFP fusion construct, the cDNA coding for dominant-negative CD99 was subcloned into the *EcoRI* sites of the EGFP expression vector, pEGFP-N_2 (BD Biosciences Clontech, San Jose, CA). The sequence of the construct was confirmed by DNA sequencing. DNA transfection to MCF-7 cells was performed using a lipofectamine reagent (Invitrogen Corp). pEGFP-N2 vector only was also transfected as a control. Stable transfectants were selected in the RPMI1640 medium, containing 10% FBS, and 0.6 mg/ml G418 (Invitrogen Corp.) for four weeks.

siRNA transfection

The siRNA duplexes were constructed with the following target sequences; human CD99, sense primer I (5'-AAGATTGCA GTGGGTTTCTTGCCTGTCTC-3') and antisense primer I (5'-AACAAGAAACCCACTGCAATCCCTGTCTC-3'); sense primer II (5'-AACATCACTGCCTCCTTTTCCCCTGTCTC-3') and antisense primer II (5'-AAGGAAAAGGAGGCAGTGATGCCTGTC TC-3'); sense primer III (5'-AACCTCCCCTTGTTCTGCATTCC TGTCTC-3') and antisense primer III (5'-AAAATGCAGAACAA GGGGAGGCCTGTCTC-3'). The construction of siRNA directed against human CD99 was carried out according to the manufacturer's instruction manual (Ambion, Austin, TX). Control siRNA was purchased from the Bioneer Corporation (Daejeon, Korea): this was the control siRNA sense primer (5'-CCUACGCCACCAAUUUCGUTT-3') and antisense primer (5'-ACGAAAUUGGUGGCGUAGGTT-3'). Cells were transfected with 40 nM siRNA duplexes by lipofectamine reagent (Invitrogen Corp.). After transfection, the medium was changed, and cells were maintained with fetal bovine serum for 24 h. Depletion of human CD99 was confirmed by Western blot as described earlier in the paper.

Flow cytometry

Flow cytometry used for detecting CD99 expression was carried out as described previously (36).

Adhesion assay

Adhesion assay was performed as described previously (36). Briefly, 1.5×10^5 MCF-7 cells were treated with 3 μg/ml of anti-human CD99 monoclonal antibody for 1 h, and then were transferred to 96-well plates coated with fibronectin, laminin, collagen IV and poly-L-lysine. After 1 h of incubation at 37°C under static conditions, unbound cells were removed by washing twice with 1x PBS. Cells bound to the extracellular matrix proteins were counted using haematocytometer under the light microscope.

Western blot analysis

Western blot analysis was carried out as described previously (36). A non-reducing condition, which denotes the absence of β-mercaptoethanol, was applied to detect the active form of β_1 integrin. Relative band intensity was measured using the ImageJ image analysis program (NIH, USA).

Confocal microscopy

The expression of EGFP protein was analyzed under a confocal microscope Olympus fluoview FV1000 (Olympus, Tokyo, Japan) as described previously (36). Expression of EGFP protein was analyzed under a confocal microscope (Olympus fluoview FV1000, Olympus, Tokyo, Japan).

Statistical analysis

The data are expressed as the average of the mean values obtained ± SD. Statistical significance was determined by a Student's t test using the statistical software GraphPad Prism (version 4.0). All experiments were conducted three times or more to ensure reproducible results. Representative data are shown in the figures provided.

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