EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 40, No. 3, 261-270, June 2008
DOI 10.3858/emm.2008.40.3.261

CD98 activation increases surface expression and clustering of β_1 integrins in MCF-7 cells through FAK/Src- and cytoskeleton-independent mechanisms

Sun-Mi Kim[1] **and Jang-Hee Hahn**[1,2]

[1]Department of Anatomy and Cell Biology
College of Medicine
Vascular System Research Center
Research Institute of Life Sciences
Kangwon National University
Chunchon 200-701, Korea
[2]Corresponding author: Tel, 82-33-250-8811;
Fax, 82-33-250-8811; E-mail, jhahn@kangwon.ac.kr

Accepted 19 February 2008

Abbreviations: ECM, extracellular matrix; FAK, focal adhesion kinase; FRNK, FAK related non-kinase; R-PE, R-phycoerythrin; SFM, serum-free medium; TCR, T cell receptor

Abstract

CD98, a disulfide-linked 125-kDa heterodimeric type II transmembrane glycoprotein, regulates β_1 integrin-mediated cell adhesion. However, the molecular mechanisms underlying CD98-mediated activation of β_1 integrin are presently unclear. In this study, the effects of CD98 signaling on the expression and clustering of β_1 integrin were investigated. Activation of CD98 augmented surface expression of β_1 integrin on MCF-7 cells. Cross-linking CD98 induced clustering of β_1 integrins. Inhibition of phosphorylation of focal adhesion kimase (FAK) by PP2, an inhibitor of Src family kinase, reduced cell-extracellular matrix adhesion, but not surface expression and clustering of β_1 integrin on MCF-7 cells. This result was confirmed by over-expression of dominant negative forms of FAK. In addition, phalloidin or cytochalasin D inhibited CD98-mediated induction of cell-ECM adhesion, but not surface expression and clustering of β_1 integrins. The inhibitory effects of PP2, cytochalasin D or phalloidin on CD98-stimulated cell adhesion were diminished by pretreatment of cells with Mn^{2+}, which is shown to induce conformational change of integrins. These results provide the first evidence that CD98 activation increases not only β_1 integrin affinity but also its surface expression and clustering and the latter is independent of FAK/Src and cytoskeleton.

Keywords: actins; antigens, CD98; cytoskeleton; focal adhesion kinase; integrin β_1

Introduction

Regulation of integrin activity plays a key role in not only normal cellular processes such as cell migration and growth but also pathological processes including metastasis of cancer cells. The α and β chains of integrin heterodimers are each composed of an extracellular region, a transmembrane segment and a small cytoplasmic domain. Integrins form complexes with other transmembrane proteins that can modulate their functions by inducing intracellular signaling. Several receptor molecules such as T cell receptor (TCR), chemokine receptors and CD99 have been shown previously to generate "inside-out signaling" that provokes changes in the conformation of integrin extracellular domain or clustering of integrins (Hahn *et al*., 1997; Burbach *et al*., 2007). In addition, CD47 and the tetraspan family (TM4SF) of receptors, CD9, CD63, CD81, CD151 have been shown to associate with integrins and affect integrin functions (Lindberg *et al*., 1996; Berditchevski, 2001).

Previous studies indicate that CD98 is an important regulator of integrin-mediated cell adhesion (Fenczik *et al*., 2001; Rintoul *et al*., 2002). The human CD98 protein is a disulfide-linked 125-kDa heterodimeric type II transmembrane glycoprotein composed of a glycosylated 85-kDa heavy chain (designated CD98) and a nonglycosylated 40-kDa light chain. CD98 is broadly expressed on many cell types including monocytes, thymocytes, proximal convoluted tubules in kidney, epithelial cells in esophagus and breast, sarcolemma in skeletal muscle and melanoma cells (Bellone *et al*., 1989; Dixon *et al*., 1990). CD98 functions include regulation of β_1 integrin function, as well as amino acid transport, cell fusion, and proliferation (Deves and Boyd, 2000). CD98 activation increased β_1 integrin-mediated adhesion of the small cell lung-cancer cell line (SCLC) H345 and breast cancer cells to fibronectin and laminin by inducing the conformational changes of β_1 integrins (Fenczik *et al*., 1997; Chandrasekaran *et al*., 1999). β_1 integrin-blocking antibodies inhibited CD98 signaling in human peripheral T lymphocytes, which may reflect convergent

signaling mechanisms between β_1 integrin and CD98 (Warren *et al*., 2000). This convergent signaling mechanism between integrins and CD98 is supported by recent findings that CD98 is associated with integrins (Zent *et al*., 2000; Fenczik *et al*., 2001), and that cross-linking CD98 induced the clustering of β_1-integrins and integrin-like signaling (Rintoul *et al*., 2002). Taken together, these results suggest that CD98 regulates β_1 integrin functions. However, its underlying molecular mechanisms remain elusive.

In this study, we investigated whether CD98 regulates the function of β_1 integrin by increasing the total number of β_1 integrin on the surface, or forming higher order multimers or clusters and whether the effect of CD98 stimulation on cell adhesion is mediated by FAK phosphorylation and cytoskeleton reorganization. The activation of CD98 molecules resulted in augmentation of cell adhesion and surface levels or clustering of β_1 integrins on MCF-7 cells. Inhibition of phosphorylation of FAK or cytoskeleton reorganization diminished CD98-induced adhesion of MCF-7 cells to fibronectin, but not CD98-induced surface expression and clustering of β_1 integrins.

Materials and Methods

Reagents

Culture reagents and Lipofectamine were purchased from Invitrogen (Carlsbad, CA). Mouse anti-human CD98 mAb (UM7F8) was purchased from BD Biosciences (San Jose, CA). Horseradish-conjugated goat anti-mouse IgG and purified goat anti-mouse IgG were from DiNona (Seoul, Korea). Anti-FAK rabbit polyclonal antibody (C-20) and anti-phophotyrosine mAb (PY99) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse anti-CD29 (β_1 integrin) mAb (3S3) and FITC-conjugated mouse anti-β_1 integrin mAb (S3S) was from Serotec Inc. (Raleigh, NC). R-PE-conjugated mouse anti-β_1 integrin mAb (TDM29) was from Cymbus Biotechnology (Eastleigh, Hampshire, UK). Cytochalasin D and phalloidin were from Tocris bioscience (Avonmouth, UK). Mouse IgG, fibronectin and phalliodin-FITC were purchased from Sigma-Aldrich (St. Louis, MO). PP2 was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA).

Cell culture

MCF-7 human breast adenocarcinoma cell line was obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 10 μg/ml streptomycin and 100 mM sodium pyrubate, 5 mg/ml insulin. Cell cultures were maintained and incubated in 5% CO_2 at 37°C.

Flow cytometric analysis

Flow cytometric analysis used for detecting β_1 integrin expression was carried out as described previously (Choi *et al*., 2007).

Stable transfection

MCF-7 cells grown to 70-80% confluence were transfected with the pcDNA3 vector (Invitrogen) encoding FAK Y397F mutant or FAK related non-kinase (FRNK) by using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The expression vector pcDNA3-FAK Y397F and pCDNA3-FRNK were kindly provided by Dr. Soo- Chul Park (Sookmyung Women's University). pcDNA3 vector was also transfected as a control. Neomycin- resistant clones were isolated by growth in DMEM containing 10% FBS and 0.8 mg/ml G418 (Invitrogen). Stable transfectant clones were selected by immunoblotting.

Adhesion assay

Nonspecific binding sites were blocked with 1% BSA in PBS for 1 h at room temperature. 2 μg of fibronectin in PBS was coated on 96-well microtiter plates overnight at 4°C. 5×10^4 MCF-7 cells in the 100 μl of serum free medium were allowed to adhere to fibronectin-coated 96-well plates, after incubation with mouse IgG (3 μg) and goat anti-mouse IgG (30 μg), or anti-CD98 mAb (3 μg) and goat anti-mouse IgG (30 μg) for 1 h. After washing with PBS three times, cell attachment was determined by counting the number of attached cells. The effects of PP2, cytochalasin D, phalloidin or over-expression of dominant-negative FAK mutants on CD98-induced cell-ECM adhesion were examined in the presence or absence of 0.5 μM $MnCl_2$ as described above.

Immunoprecipitation and Western blot analysis

MCF-7 cells were lysed in protein lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10 μg/ml aprotinin, 100 mM sodium orthovanadate, 1 mM PMSF). FAK was precipitated from the cell lysates with rabbit anti-FAK polyclonal antibody overnight at 4°C. Protein-antibody complexes were incubated with either protein A agarose beads for 3 h at 4°C. The precipitates were washed with Tris-buffered saline/Tween (20 mM

Tris-HCl pH 7.4, 150 mM NaCl, and 0.05% [vol/vol] Tween 20) and boiled in SDS sample buffer for 10 min before loading on 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, blocked using 3% (wt/vol) albumin in Tris-buffered saline/Tween overnight at 4°C and then incubated with anti-FAK or anti-phospho-tyrosine antibody. HRP-conjugated antibodies were used for secondary labeling. Immunoreactive bands were identified using Westzol (Intron, Seoul, Korea) according to the manufacturer's instruction.

Determination of integrin clustering

5×10^5 MCF-7 cells were plated and grown onto glass cover-slips coated with fibronectin (20 μg/ml) for 24 h. After washing with SFM twice, cells were incubated with mouse IgG (30 μg/ml), or anti-CD98 mAb UM7F8 (30 μg/ml), or anti-β_1 integrin mAb 3S3 (30 μg/ml) for 1 h at room temperature. When cross-linking is necessary, goat anti-mouse IgG (300 μg/ml) was added as secondary antibody. After washing twice with PBS, cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, they were permeabilized in 0.1% Triton x-100. Permeabilized cells were incubated with 1 μg of R-PE-conjugated mouse anti-β_1 integrin mAb TDM29 and phalloidin-FITC for 1 h at room temperature. Finally, samples were washed, mounted onto slides, embedded with mounting medium (DakoCytomation, Carpinteria, CA), and analyzed under a confocal microscope Zeiss LSM510 META NLO (Carl Zeiss, Bernried, Germany). Images were processed using the Adobe Photoshop software. Signal intensities were measured by using LSM 510 Meta NLO software (Carl Zeiss, Bernried, Germany).

Statistical analysis

The data are expressed as the average of the mean values obtained ± SE. Statistical significance was determined as described previously (Lee *et al*., 2006).

Results

Cross-linking CD98 elevates the surface expression of β_1 integrin

Integrin affinity for ECM ligands was up-regulated by the activated CD98 (Fenczik *et al*., 1997; Chandrasekaran *et al*., 1999). Enhanced cell adhesion could result from the elevated expression of cellular adhesion molecules. For this reason, we examined the effect of CD98 activation on expression of β_1 integrin on MCF-7 cells. Flow cytometry analysis showed cell surface expression of β_1 integrin significantly increased in MCF-7 cells stimulated by anti-CD98 mAb (Figure 1A). However, Western blot analysis showed that CD98 activation did not have any effect on the total expression of β_1 integrin in MCF-7 cells, suggesting that CD98 activation may be involved in exocytosis rather than synthesis of β_1 integrin (Figure 1B).

Cross-linking of CD98 induces clustering of β_1 integrin

Previous studies demonstrated that cross-linking CD98 induced clustering of β_1 integrins (Kolesnikova *et al*., 2001). To determine the underlying mechanisms by which CD98 cross-linking induces clustering of β_1 integrins on the cell surface, we

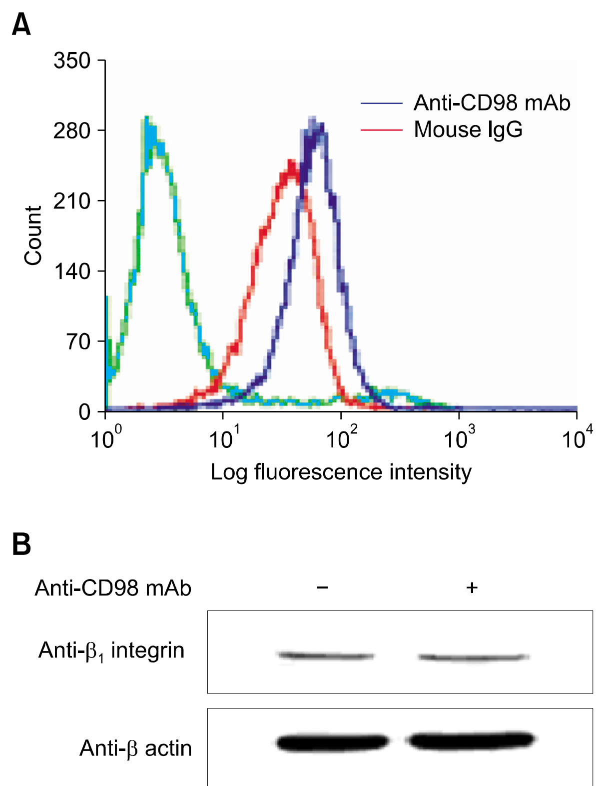
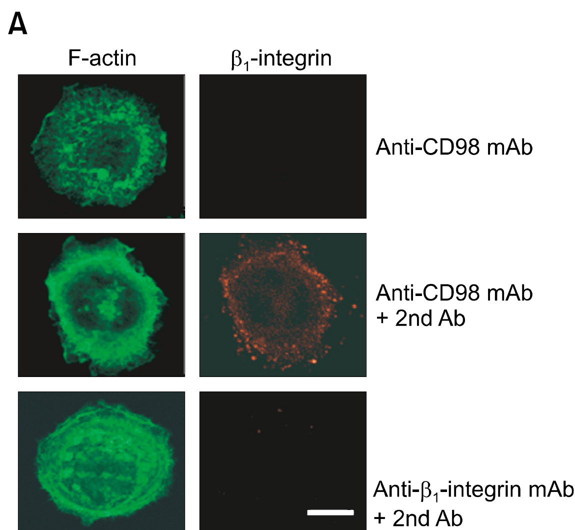


Figure 1. CD98 engagement increases the surface expression of β_1 integrin. (A) Freshly cultured MCF-7 cells were dispersed and then incubated with anti-CD98 mAb UM7F8 and secondary Ab (blue line) for 1 h. As negative controls, cells were treated with mouse IgG and secondary Ab (red line). FITC-conjugated anti-CD29 mAb was used to measure the expression level of β_1 integrin on the cells treated with mAbs as described above. An FITC-conjugated mouse anti-human IgG was used as the negative control (green line). (B) Cell extracts from MCF-7 cells treated as in (A) were analyzed by Western blotting using anti-β_1 integrin and anti-actin mAb.

compared the cell surface distribution of β_1 integrins following CD98 or β_1 integrin engagement on MCF-7 cells. Confocal microscopy analysis revealed that cross-linking with anti-CD98 mAb induced the formation of multiple small patches of β_1 integrin on the cell surface much more than that with anti-β_1 integrin mAb (Figure 2A and B). Clustering of β_1 integrins was strictly dependent on cross-linking, because without a secondary Ab, β_1 integrin clustering was not observed on the cell surface. Cross-linking with mouse IgG and secondary Ab had no effect on clustering of β_1 integrins, supporting the specificity of the phenomenon further (data not shown). These observations suggest that cross-linking CD98 facilitates β_1 integrin clustering on the surface of MCF-7 cells by triggering signals to enhance clustering actively.



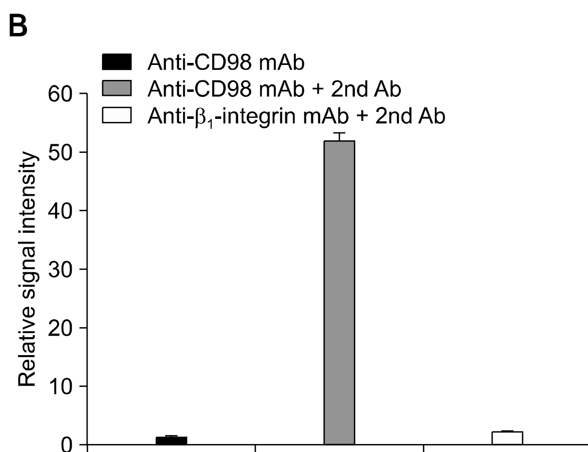


Figure 2. CD98 cross-linking enhances β_1 integrin clustering. (A) MCF-7 cells were incubated with anti-CD98 mAb UM7F8 with or without secondary antibody. To determine whether cross-linking of β_1 integrins induces their clustering, cells were treated with anti-β_1 integrin mAb 3S3 in the presence of secondary antibody as well. Next, cells treated as described in "Materials and Methods" were analyzed by confocal microscopy. Actin cytoskeleton organization is visualized by staining with phalloidin-FITC whereas β_1 integrin clustering with R-PE-conjugated anti-β_1 integrin mAb. Images are from a single experiment representative of more than three so performed. Scale bar, 50 μm. Original magnification, × 400. (B) Relative intensities of β_1 integrin clusters in Figure 2A were measured with LSM5120 Meta NLO software. Results are values relative to the β_1 integrin signal intensity level of untreated controls, designated as 1.

Pharmacologic inhibition of FAK/Src kinase activity blocks CD98-induced cell adhesion

Previous study showed overexpression or cross-linking of CD98 led to an increase in FAK phosphorylation (Rintoul *et al*., 2002; Cai *et al*., 2005). We determined whether FAK phosphorylation is required for CD98-induced adhesion of MCF-7 cells to ECM. FAK phosphorylation in MCF-7 cell cultures after CD98 engagement was examined by immunoprecipitation and immunoblotting. Figure 3A showed crosslinking CD98 triggered FAK autophosphorylation in suspended MCF-7 cells before adhesion to matrix. This elevated phosphorylation of FAK was significantly reduced by the pretreatment with PP2 (0.2 μM), an inhibitor of Src family kinase (Salazar and Rozengurt, 2001), with no inhibitory effect due to the PP2 solvent DMSO (Figure 3A, lanes 2 and 3). To investigate effects of FAK phosphorylation on the CD98-mediated cell adhesion, we treated MCF-7 cells with anti-CD98 mAb in the presence or absence of PP2 (0.2 μM) and then the effect of PP2 on the adhesion of MCF-7 cells to fibronectin was examined. Figure 3B shows inhibition of CD98-induced cell adhesion to fibronectin by PP2. These results suggest that FAK/Src kinase activity may be required for CD98-stimulated cell adhesion. In contrast, inhibition of FAK phosphorylation with pretreatment of cells with PP2 (0.2 μM) has no effect on surface expression and clustering of β_1 integrins, suggesting that CD98 activation upregulates surface expression and clustering of β_1 integrin through FAK-independent pathway (Figure 3C and D).

Next, we examined whether conformational change of β_1 integrin with $MnCl_2$ (0.5 μM) prevents the effect of PP2 on CD98-stimulated cell adhesion. Addition of $MnCl_2$ diminished the effect of PP2 on the CD98-induced cell adhesion (Figure 3B). However, conformational activation of β_1 integrin by Mn^{2+} is not associated with FAK (Figure 3A, bottom). These data are consistent with the previous report showing that addition of Mn^{2+} did not affect FAK or Src phosphorylation, but increased cell-ECM adhesion (Thamilselvan and Basson, 2004).

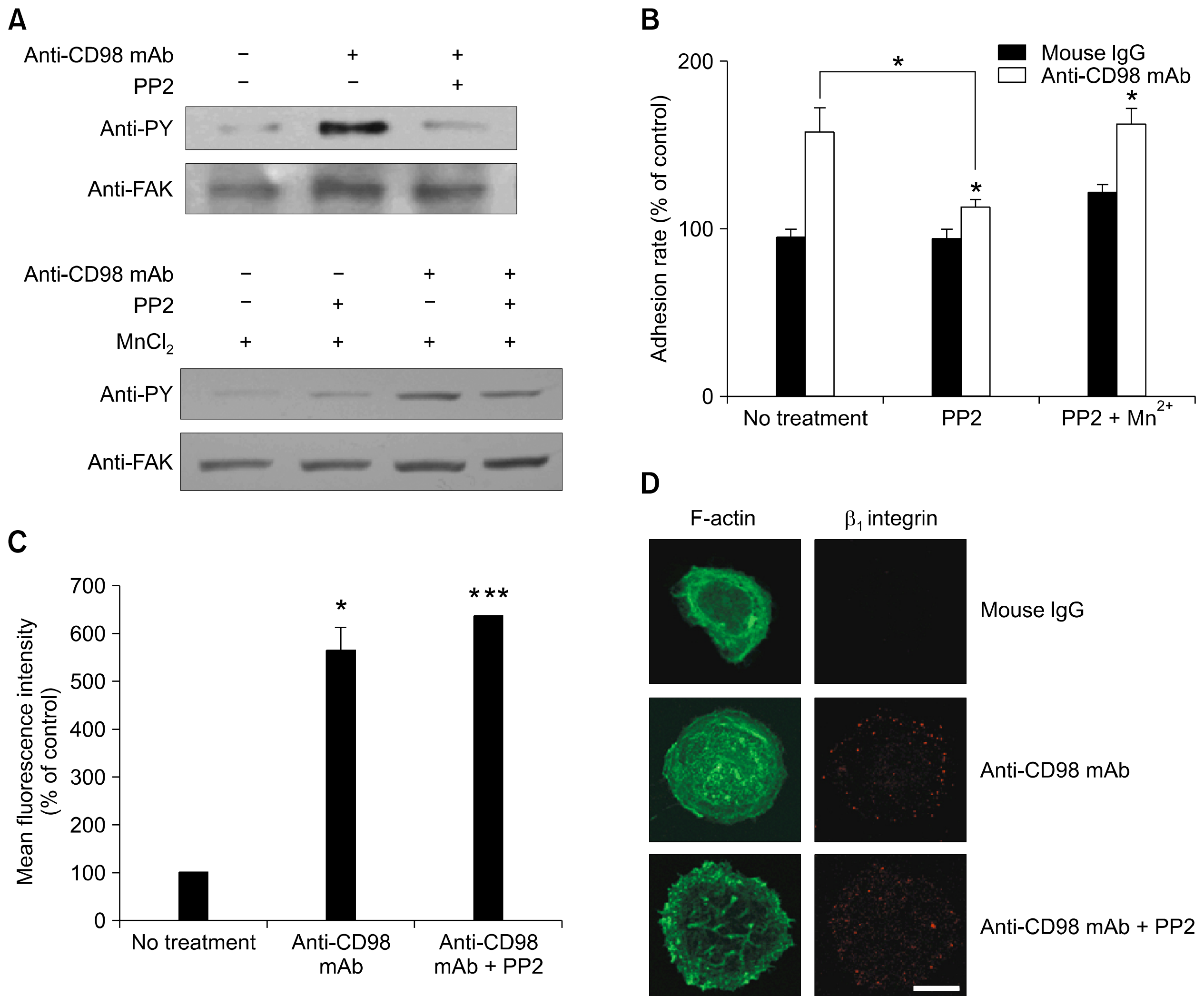


Figure 3. Inhibition of FAK/Src kinases with PP2 blocks CD98-induced cell adhesion, but not surface expression and clustering of β_1 integrins in MCF-7 cells. (A) The effect of PP2 and/or Mn^{2+} on FAK phosphorylation in MCF-7 cells treated with anti-CD98 mAb was determined by immunoprecipitaion assay. MCF-7 cells were incubated with anti-CD98 mAb and PP2 (0.2 μM) or a DMSO vehicle control in the presence or absence of 0.5 μM Mn^{2+} for 1 h and anti-FAK rabbit polyclonal antibody (C-20) was used to immunoprecipitate FAK from extracts of MCF-7 cells. Immunoprecipitates were blotted and probed with anti-phosphotyrosine mAb (clone PY99) and anti-FAK mAb. (B) The effect of PP2 treatment on cell adhesion rate was determined as described in Materials and Methods. In addition, whether addition of 0.5 μM Mn^{2+} interferes with the effect of PP2 on cell adhesion was determined by the same way. Results are expressed as mean ± SE of values relative to the adhesion rate of mouse IgG-treated controls, designated as 100%. Asterisks show a significant difference from control as follows: $^*P < 0.05$. Additional statistical comparisons are indicated by lines. (C) MCF-7 cells were incubated with anti-CD98 mAb with or without PP2 (0.2 μM) and then analyzed by flow cytometry using FITC-conjugated anti-human β_1 integrin mAb. Data represent the mean ± SE of values relative to mean values of fluorescence intensity of mouse IgG-treated controls, designated as 100%. Asterisks show a significant difference from control as follows: $^*P < 0.05$, $^{***}P < 0.001$ (D) Confocal microscopy was performed as described in Figure 2 legend to investigate the effect of PP2 (0.2 μM) on CD98-induced clustering of β_1 integrins. Scale bar, 50 μm. Original magnification, × 400.

Over-expression of dominant-negative mutant FAK diminishes the effect of CD98 activation on cell adhesion

Because protein kinase inhibitors have the potential to target protein kinases nonspecifically, we confirmed the effect of FAK on the CD98-mediated cell adhesion by overexpression of dominant-negative forms of FAK in MCF-7 cells. We compared the effect of CD98 activation on adhesion of mock transfectants and cells transfected with dominant-negative forms of FAK that appeared to decrease integrin affinity (Thamilselvan and Basson, 2004). The cell lines that stably overexpressed the kinase-deficient FAK (FRNK or Y397F-FAK) were identified by Western blot analysis (Figure 4A). Transfected cells were treated with anti-CD98 mAb for 1 h, and then replated on fibronectin. As shown

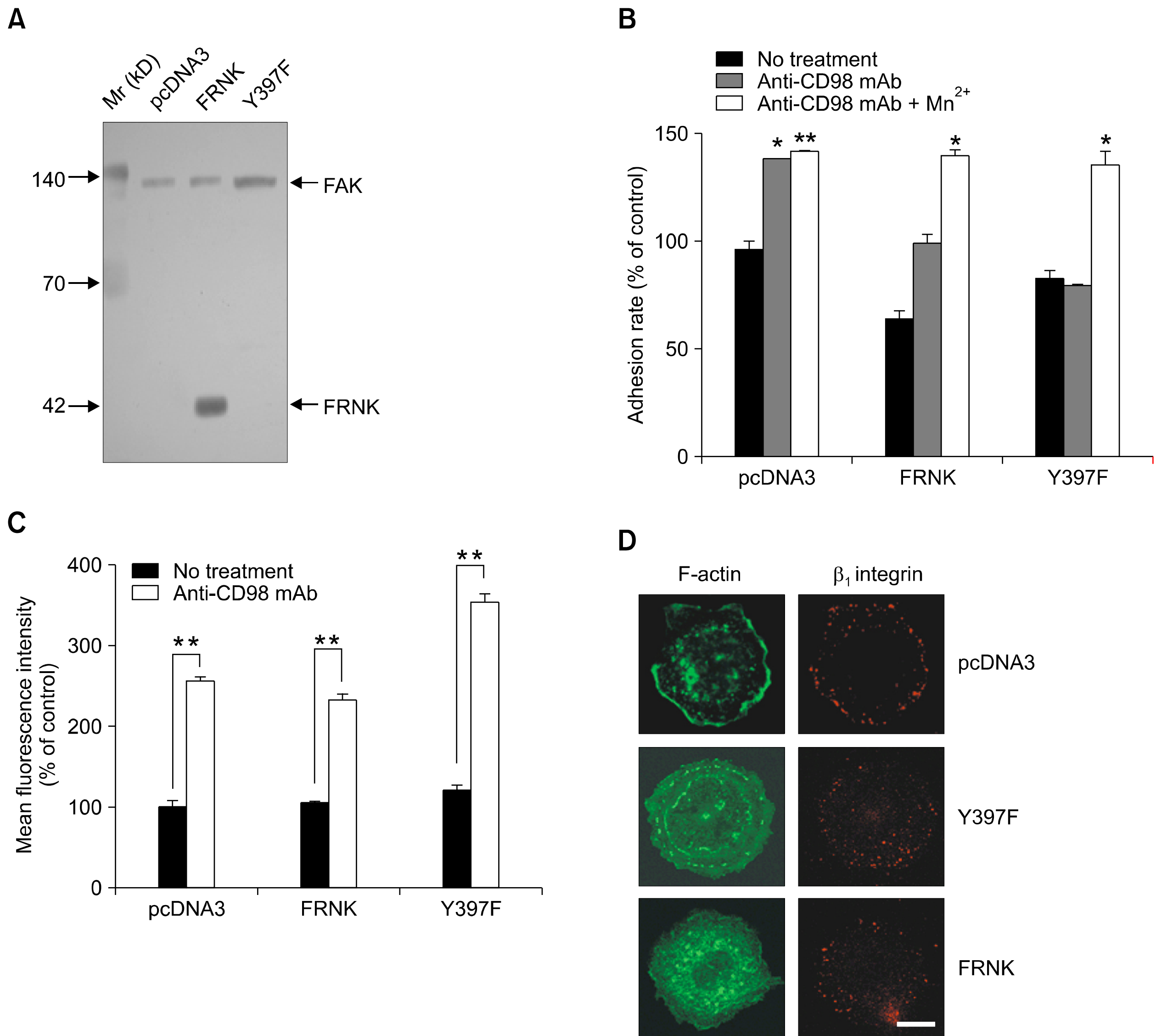


Figure 4. The effects of dominant-negative variants of FAK on adhesiveness of MCF-7 cells, surface expression and clustering of β_1 integrins. (A) MCF-7 cells were stably transfected with dominant-negative mutant FAK constructs (FRNK, Y397F-FAK) or control vector, pcDNA3. The expression levels of endogenous FAK, FRNK and Y397F-FAK were determined by Western blot analysis using anti-FAK polyclonal antibody. (B) The effect of dominant-negative variants of FAK on CD98-induced cell adhesion rate was determined as described in Materials and Methods. In addition, whether addition of 0.5 μM Mn^{2+} interferes with the effect of PP2 on cell adhesion was determined by the same way. Results are expressed as in Figure 3 (B). $^*P < 0.05$, $^{**}P < 0.01$ (C) FAK variants- or mock-transfected MCF-7 cells were treatred with anti-CD98 mAb and secondary antibody and then analyzed for expression of β_1 integrin through flow cytometry using FITC-conjugated anti-human β_1 integrin. Results are expressed as in Figure 3 (C). Statistical comparisons are indicated by lines. $^{**}P < 0.01$ (D) Confocal microscopy was performed as described above to investigate the effect of dominant-negative variants of FAK on clustering of β_1 integrins. Scale bar, 50 μm. Original magnification, × 400.

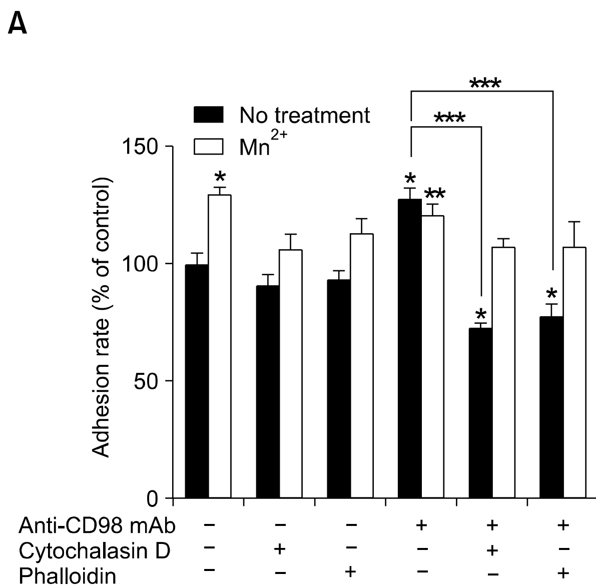
in Figure 4B, FRNK and Y397F-FAK-transfected cells did not increase adhesion in response to CD98 stimulus that increased adhesion of the untransfected cells as well as that of mock-transfected cells. Addition of Mn^{2+} to the dominant-negative mutant FAK clones restored cell adhesion. These results are consistent with the above results indicating that CD98 signaling increases cell adhesion through phosphorylation of FAK. In contrast, high-level expression of the FAK mutants did not have any effect on CD98-induced surface expression or clustering of β_1 integrins (Figure 4C and D). As a whole, these experiments indicate that FAK phosphorylation is required for CD98-induced adhesion of MCF-7 cells to extracellular matrix, but not for surface expression and clustering of β_1 integrins.

CD98-stimulated adhesion is blocked by actin cytoskeletal inhibition

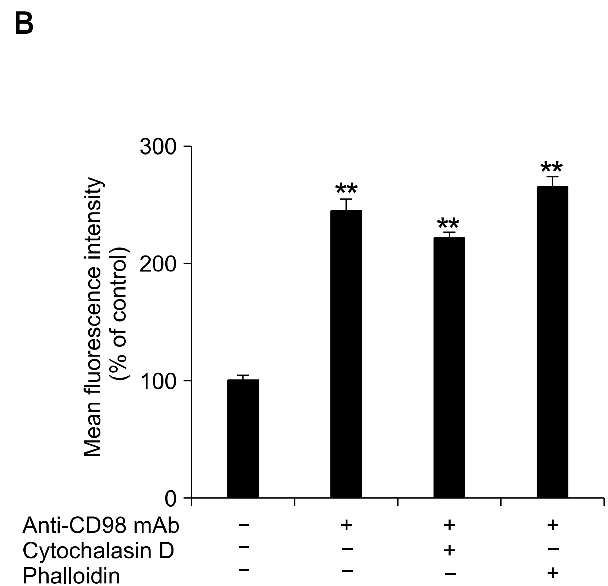
Because the activation of FAK and Src leads to the reorganization of cytoskeleton, we investigated the role of actin cytoskeleton for CD98-stimulated cell adhesion. To investigate whether disrupting actin polymerization or reorganization could inhibit CD98-induced cell adhesion, CD98 were cross-linked in the presence of 4 μM cytochalasin D or 10 μM phalloidin. Pretreatment of MCF-7cells with cytochalasin D or phalloidin inhibited CD98-stimulated MCF-7 cell adhesion to fibronectin (Figure 5A). However, cytochalasin D or phalloidin-treated cells strongly adhered to fibronectin when treated with 0.5 μM $MnCl_2$, indicating that β_1 integrins on those cells are potentially functional, but cytochalasin D or phalloidin treatment inhibits inside-out activation of β_1 integrins or stabilization of adhesion complex through actin cytoskeleton.

Next, we investigated whether cytoskeletal reorganization is required for CD98-mediated upregulation of clustering and surface expression of β_1 integrins. Flow cytometric analyses revealed that cytochalasin D and phalloidin had no effects on the

A



B



C

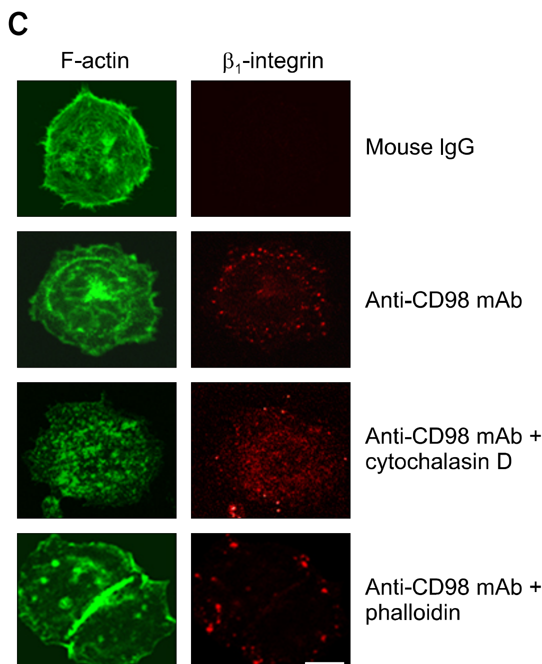


Figure 5. Cytochalasin D or phalloidin treatment inhibits CD98-induced adhesion of MCF-7 cells to fibronectin, but not surface expression and clustering of β_1 integrins in MCF-7 cells. (A) MCF-7 cells were incubated with anti-CD98 mAb in the presence or absence of cytochalasin D (4 μM) or phalloidin (10 μM) for 1 h. The effects of DMSO vehicle control are also shown. The effect of cytochalasin D treatment on cell adhesion was determined as described in Materials and Methods. In addition, whether addition of 0.5 μM Mn^{2+} interferes with the effect of cytochalasin D or phalloidin on cell adhesion was determined by the same way. Results are expressed as Figure 3B. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (B) MCF-7 cells treated with anti-CD98 mAb in the presence or absence of cytochalasin D or phalloidin were analyzed for expression of β_1 integrin using flow cytometry. Results are expressed as in Figure 3 (C). $^{**}P < 0.01$ (C) Confocal microscopy was performed as described above to investigate the effect cytochalasin D or phalloidin of on clustering of β_1 integrins. Scale bar, 50 μm. Original magnification, × 400.

upregulation in expression levels of β_1 integrins by CD98 activation (Figure 5B). Confocal analysis showed that blocking actin rearrangement had no effect on clustering of β_1 integrins induced by cross-linking CD98 (Figure 5C). Taken together, these results indicate that actin polymerization is necessary for the CD98-induced adhesion of MCF-7 cells to extracellular matrix proteins, but may not be important for the surface expression and clustering of β_1 integrins.

Discussion

In this study, we have examined the effect of CD98 activation on adhesion of MCF-7 human breast carcinoma cells to ECM. We report that: 1) cross-linking CD98 increases surface expression and clustering of β_1 integrins as well as their affinity in MCF-7 cells. 2) inhibition of CD98-induced FAK phosphorylation or blocking actin rearrangement does not prevent surface expression and clustering of β_1 integrins but their conformational changes, which leads to reduction of cell adhesion.

Previous studies have demonstrated that CD98 activation leads to up-regulation of β_1 integrin activity (Fenczik *et al*., 1997; Cai *et al*., 2005). The underlying mechanisms could include up-regulation in the cell surface expression levels of β_1 integrin by exocytosis, its avidity by clustering, or its affinity by conformational changes. Our results suggest that some combination of mechanisms described above may be involved in CD98-mediated regulation of β_1 integrin activity, although it is difficult to determine the relative contributions of each (Hato *et al*., 1998).

Our flow cytometry analysis showed that cell surface expression of β_1 integrin was enhanced by CD98 activation, suggesting that CD98 upregulates the activity of β_1 integrin not only by inducing conformational changes (Chandrasekaran *et al*., 1999; Cai *et al*., 2005), but also by augmenting cell surface expression of active β_1 integrin quantitatively. CD98 stimulation increased the expression of β_1 subunit at cell surface in a relatively short time. In addition, Western blot analysis showed that CD98 activation did not have any effect on the total expression of β_1 integrin in MCF-7 cells, suggesting that CD98 activation may be involved in transport from endocytic vesicles to cell surface rather than *de novo* synthesis of β_1 integrin. This result is consistent with the observation that surface expression of CD98 increases in a human placental trophoblast cell line (BeWo) in the presence of CD98 mAb (Dalton *et al*., 2007).

According to our confocal microscopy analysis, cross-linking CD98 induces clustering of β_1 integrins on MCF-7 cells. This is supported by the results showing that CD98 engagement led to formation of round clusters of β_1 integrins (Kolesnikova *et al*., 2001; Rintoul *et al*., 2002). Clustering of integrins has been known to be a well-established mechanism to enhance integrin mediated adhesion (Jaakkola *et al*., 2003). Since CD98 specifically associates with β_1 integrins (Zent *et al*., 2000; Fenzik *et al*., 2001; Miyamoto *et al*., 2003), clustering might occur passively as a consequence of physical associations of integrins with CD98 without regard to cytoskeleton. However, this study showed that cross-linking CD98 induces more clustering of β_1 integrin than cross-linking β_1 integrin on MCF-7 cells, suggesting that CD98-induced clustering of β_1 integrins could not result from simple secondary antibody-mediated cross-linking of CD98 and its subsequent clustering of β_1 integrins. Alternatively, CD98 activation could induce actin reorganization and the associated distribution of integrins through inside-out signaling. Cross-linking of Ly6, a hemopoietic cell differentiation antigen found on a subset of CD8 T cells in the periphery, could trigger a signal for cytoskeletal reorganization and clustering of LFA-1 (Jaakkola *et al*., 2003). Phalloidin or cytochalasin D treatment did not inhibit CD98-induced clustering of β_1 integrins, suggesting that cross-linking of CD98 causes clustering of β_1 integrins on the surface of MCF-7 cells via a mechanism independent of reorganization of actin cytoskeleton. Interestingly, the same treatment inhibited the effects of CD98 on cell adhesion, but not surface expression and clustering of β_1 integrins. These results indicate that increased surface expression and clustering of β_1 integrins is not sufficient for CD98-induced cell adhesion.

Our data demonstrate that FAK, Src and actin cytoskeleton are required for CD98-induced cell adhesion, but not for surface expression and clustering of β_1 integrins. Previous studies showed that cross-linking CD98 increased phosphorylation of FAK dependent on β_1 integrin-mediated signaling pathway (Rintoul *et al*., 2002; Cai *et al*., 2005). Our study showed that CD98-induced cell adhesion was significantly reduced by the pretreatment with PP2. These results were confirmed by over-expression of dominant negative forms of FAK in this study and consistent with previous report (Rintoul *et al*., 2002). Furthermore, cytochalasin D or phalloidin inhibited CD98-induced cell adhesion. PP2-, cytochalasin D- or phalloidin-treated cells strongly adhered to fibronectin when treated with 0.5 μM $MnCl_2$, indicating that β_1 integrins on those cells are potentially functional. It has been proposed that FAK-, Src-, and actin cytoskeleton-dependent sig-

naling could play a role in direct induction of a conformational change of β_1 subunit (Gomez-Rodriquez *et al.*, 2007; Thamilselvan *et al.*, 2007) or maintenance of talin activity (Cram and Schwarzbauer, 2004). Thus, CD98 signals may induce conformational changes in β_1 integrin through phosphorylation of FAK and reorganization of cytoskeleton. Alternatively, CD98 activation might modulate the affinity of β_1 integrin by directly inducing conformational changes, because CD98 physically associates with β_1 integrins. Previously, it was shown that thrombospondin-bound integrin associated protein (CD47) physically and functionally modifies integrin $\alpha_{IIb}\beta_3$ by its extracellular domain rather than traditional inside-out signaling (Fujimoto *et al.*, 2003). In this case, FAK phosphorylation and subsequent actin reorganization may not be involved in the activation of β_1 integrins, but in the stabilization of adhesion structure (Mitra *et al.*, 2005; Alon and Dustin, 2007). It remains to be addressed whether CD98-induced FAK phosphorylation and actin cytoskeletal reorganization could directly increase β_1 integrin affinity or simply the stability of adhesion complex.

In summary, we demonstrate that CD98 activation leads to an increase in surface expression and clustering of β_1 integrins, and that FAK, Src, and a functional actin cytoskeleton are required for CD98-induced cell adhesion to matrix. It remains elusive whether increase in surface expression of β_1 integrins causes enhancement of cell adhesion, and whether binding of cognate ligands for CD98 actually will induce clustering of β_1 integrins, which will increase binding avidity. In addition, it is not clear whether or not CD98 stimulates β_1 integrin affinity by an inside-out signaling pathway that requires FAK, Src, and a functional actin cytoskeleton. These issues should be addressed for further understanding the molecular mechanism of CD98 functions.

Acknowledgements

We thank Min-Kyoung Kim, Si-Eun Kim, Kyoung-Jin Lee and Seung Hae Kwon for their technical help. This work was supported by Vascular System Research Center grant from the Korea Science and Engineering Foundation.

References

Alon R, Dustin ML. Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen-presenting cells. Immunity 2007;26:17-27

Bellone G, Alloatti G, Levi R, Geuna M, Tetta C, Peruzzi L, Letarte M, Malavasi F. Identification of a new epitope of the 4F2/44D7 molecular complex present on sarcolemma and isolated cardiac fibers. Eur J Immunol 1989;19:1-8

Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. J Cell Sci 2001;114:4143-51

Burbach BJ, Medeiros RB, Mueller KL, Shimizu Y. T-cell receptor signaling to integrins. Immunol Rev 2007;218: 65-81

Cai S, Bulus N, Fonseca-Siesser PM, Chen D, Hanks SK, Pozzi A, Zent R. CD98 modulates integrin β1 function in polarized epithelial cells. J Cell Sci 2005;118:889-99

Chandrasekaran S, Guo NH, Rodrigues RG, Kaiser J, Roberts DD. Proadhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by integrin and regulated by insulin-like growth factor-1 and CD98. J Biol Chem 1999;274:11408-16

Choi G, Lee SK, Jung KC, Choi EY. Detection of homodimer formation of CD99 through extracelluar domain using bimolecular fluorescence complementation analysis. Exp Mol Med 2007;36:746-55

Cram EJ, Schwarzbauer JE. The talin wags the dog: new insights into integrin activation. Trends Cell Biol 2004; 14:55-7

Dalton P, Christian HC, Redman CW, Sargent IL, Boyd CA. Differential effect of cross-linking the CD98 heavy chain on fusion and amino acid transport in the human placental trophoblast (BeWo) cell line. Biochim, Biophys, Acta 2007; 768:401-10

Deves R, Boyd CA. Surface antigen CD98 (4F2): not a single membrane protein, but a family of proteins with multiple functions. J Membr Biol 2000;173:165-77

Dixon WT, Sikora LK, Demetrick DJ, Jerry LM. Isolation and characterization of a heterodimeric surface antigen on human melanoma cells and evidence that it is the 4F2 cell activation/proliferation molecule. Int J Cancer 1990;45: 59-68

Fenczik CA, Sethi T, Ramos JW, Hughes PE, Ginsberg MH. Complementation of dominant suppression implicates CD98 in integrin activation. Nature 1997;390:81-5

Fenczik CA, Zent R, Dellos M, Calderwood DA, Satriano J, Kelly C, Ginsberg MH. Distinct domains of CD98hc regulate integrins and amino acid transport. J Biol Chem 2001;276: 8746-52

Fujimoto TT, Katsutani S, Shimomura T, Fujimura K. Thrombospondin-bound integrin-associated protein (CD47) physically and functionally modifies integrin $\alpha_{IIb}\beta_3$ by its extracellular domain. J Biol Chem 2003;278:26655-65

Gomez-Rodriquez J, Readinger JA, Viorritto IC, Mueller KL, Hoightling RA, Schwartzberg PL. Tec kinases, actin, and cell adhesion. Immunol Rev 2007;218:45-64

Hahn JH, Kim MK, Choi EY, Kim SH, Sohn HW, Ham DI, Chung DH, Kim TJ, Lee WJ, Park CK, Ree HJ, Park SH. CD99 (MIC2) regulates the LFA-1/ICAM-1-mediated adhesion of lymphocytes, and its gene encodes both positive and negative regulators of cellular adhesion. J Immunol 1997; 159:2250-8

Hato T, Pampori N, Shattil SJ. Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin αIIbβ3. J Cell Biol

1998;141:1685-95

Jaakkola I, Merinen M, Jalkanen S, Hänninen A. Ly6C Induces Clustering of LFA-1 (CD11a/CD18) and is involved in subtype-specific adhesion of CD8 T cells. J Immunol 2003;170:1283-90

Kolesnikova TV, Mannion B, Berditchevski F, Hemler ME. β1 integrins show specific association with CD98 protein in low density membranes. BMC Biochem 2001;2:10-8

Lee KJ, Kim YM, Kim DY, Jeoung D, Han K, Lee ST, Lee YS, Park KH, Park JH, Kim DJ, Hahn JH. Release of heat shock protein 70 (Hsp70) and the effects of extracellular Hsp70 on matric metalloproteinase-9 expression in human monocytic U937 cells. Exp Mol Med 2006;38:364-74

Lindberg FP, Gresham HD, Reinhold MI, Brown EJ. Integrin-associated protein immunoglobulin domain is necessary for efficient vitronectin bead binding. J Cell Biol 1996;134:1313-22

Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol 2005;6:56-68

Miyamoto YJ, Mitchell JS, McIntyre BW. Physical association and functional interaction between β_1 integrin and CD98 on human T lymphocytes. Mol Immunol 2003;39: 739-51

Rintoul RC, Buttery RC, Mackinnon AC, Wong WS, Mosher D, Haslett C, Sethi T. Cross-linking CD98 promotes integrin-like signaling and anchorage-independent growth. Mol Biol Cell 2002;13:2841-52

Salazar EP, Rozengurt E. Src family kinases are required for integrin-mediated but not for G protein-coupled receptor stimulation of focal adhesion kinase autophosphorylation at Tyr-397. J Biol Chem 2001;276:17788-95

Thamilselvan V, Basson MD. Pressure activates colon cancer cell adhesion by inside-out focal adhesion complex and actin cytoskeletal signaling. Gastroenterology 2004; 126;8-18

Thamilselvan V, Craig DH, Basson MD. FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway. FASEB J 2007;21:1730-41

Warren AP, Patel K, Miyamoto Y, Wygant, JN, Woodside DG, McIntyre BW. Convergence between CD98 and integrin-mediated T-lymphocyte co-stimulation. Immunology 2000; 99:62-8

Zent R, Fenczik CA, Calderwood DA, Liu S, Dellos M, Ginsberg MH. Class-and splice variant-specific association of CD98 with integrin β cytoplasmic domains. J Biol Chem 2000;275;5059-64