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Functional involvement of src and focal adhesion kinase in a CD99 splice variant-induced motility of human breast cancer cells

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Abbreviations: FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; mAb, monoclonal antibody

Abstract

Earlier report showed that expression of a splice variant of CD99 transmembrane protein increases invasive ability of human breast cancer cells. Cell motility was also significantly enhanced by the CD99 splice variant expression. In an effort to identify the cellular components that mediate a signal transduction pathway tiggered by the CD99 splice variant, known signal path inhibitors were examined for their effects on the motility of the CD99 splice variant-transfected MDA-MB-231 breast cancer cells. Phenylarsine oxide, an inhibitor of phosphatase specific for focal adhesion kinase, and PP1, an inhibitor of src kinase family, significantly suppressed motility of the cells. Among different types of *src* transfectant clones generated, kinase-negative mutant *src* transfectant cells were 80% less motile than the mock cells transfected with an empty-vector, while *v-src* and *c-src* transfectants exhibited cell motility levels at or slightly above the mock transfectant. These results suggest that src and focal adhesion kinase mediate the intracellular signaling pathway of a CD99 splice variant for the induction of motility of human breast cancer cells.

Keywords: breast cancer, CD99, focal adhesion kinase, motility, splice variant, src.

Introduction

CD99 is a transmembrane glycoprotein with a molecular mass of 32 kDa encoded by the *MIC2* gene (Levy *et al*., 1979). The CD99 protein has been implicated in various cellular processes of hematopoietic cells, including homophilic cell adhesion, apoptosis, vesicular protein transport, and thymocyte differentiation (Bernard *et al*., 1995; 1997; Choi *et al*., 1998; Sohn *et al*., 2001). Although the ligand(s) for CD99 is yet to be identified, cross-linking of CD99 molecules with an anti-CD99 monoclonal antibody was shown to induce homotypic aggregation of lymphocytes, along with up-regulation of LFA-1 ($\alpha_L\beta_2$ integrin) expression, suggesting that signal transduction via CD99 modulates cell adhesion of lymphocytes by regulating the expression level of cell adhesion molecules (Bernard *et al*., 1995; Hahn *et al*., 1997). It was recently reported that the induction of homotypic aggregation of Jurkat T cells by CD99 activation is mediated by protein kinases including MAP kinases, protein tyrosine kinases, and protein kinase C (Hahn *et al*., 2000; Kasinrerk *et al*., 2000).

The CD99 gene encodes two distinct proteins, which are produced by alternative splicing of the CD99 gene transcript (Hahn *et al*., 1997). Interestingly, the minor, splicing variant form of CD99 inhibited homotypic adhesion of B cells, while activation of the major CD99 form promoted the adhesion process, indicating that the CD99 gene produces two distinct proteins with opposite functions in adhesion process of lymphocytes. Since the two forms of CD99 were shown to be differentially expressed in many human cells and highly conserved in monkey, it was suggested that the two forms of CD99 function *in vivo* in both positive and negative regulation of homophilic cell adhesion of lymphocytes during differentiation and immune responses (Hahn *et al*., 1997).

CD99 is broadly distributed on many cell types, and strongly expressed on human cortical thymocytes, Ewings sarcoma cells and peripheral primitive neuroectodermal tumors (Kovar *et al*., 1990; Ambros *et al*., 1991). Especially, the alternatively spliced isoforms of CD99 transcripts are differentially expressed in a cell type-specific manner among hematopoietic cells (Hahn *et*

al., 1997). In a previous study of the CD99 expression in human breast cancer cell lines, a highly metastatic breast adenocarcinoma cell line, MDA-MB-435 was found to express both the major form and splice variant of CD99, while a non-invasive breast adenocarcinoma cell line, MCF-7 expresses only the major CD99 form. Such results suggest that expression of the CD99 splice variant may be differentially regulated during development and/or progression of human breast cancer. Interestingly, transfection of an invasive breast adenocarcinoma cell line, MDA-MB-231 lacking both the CD99 types with an expression construct of the CD99 splice variant, significantly enhances *in vitro* invasiveness and inhibition of homophilic cell adhesion (Kim *et al*., 2000). Several studies have demonstrated the CD99 molecule as a signal transducer. Whether the CD99 variant also functions either as an inhibitor of CD99 induced cell signal path and/or an independent signal transducer to induce such cellular activities as invasion and homotypic cell adhesion is not known. In the present study, we investigated signal transduction pathway(s) downstream of the CD99 splice variant in breast cancer cells.

Materials and Methods

Cell culture

MDA-MB-231 human breast adenocarcinoma cell line was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Gibco-BRL, Grand Island, NY, USA). For cultures of the CD99 splice variant-transfected MDA-MB-231 cells, G418 was added to the complete DMEM at a concentration of 0.8 mg/ml. Cell cultures were main-tained and incubated in 5% CO_2 at 37°C.

Migration assay

Cell motility was measured by wound migration assay as described previously (Shin *et al*., 2001). Briefly, the CD99 splice variant transfectant cells (5×10^5) in 2 ml of culture medium containing 10% FBS and 0.8 mg/ml G418 were seeded in a well of 24-well culture plate. When the cells grew up to confluent state, a wound was made in the confluent monolayer with a plastic micropipette tip having large orifice. The medium and debris were aspirated away and replaced by 2 ml of fresh medium. For the evaluation, photographs of wounded area were taken just after making a wound and every 24 h after incubation, respectively. The extent of migration was quantified by measuring the migration distance of each group of cells.

Transfection experiments

The cDNA expression construct encoding c-src, v-src or dominant-negative mutant src lacking kinase activity was cotransfected with a pTK/*Hyg* (Invitrogen, Carlsbad, CA, USA) into the CD99 splice variant-transfected MDA-MB-231 breast cancer cells by using lipofectAMINE (Gibco-BRL) according to the manufacturer's instructions. PTK/*Hyg* vector only was also transfected as a control. Src transfectants were selected in DMEM containing 10% FBS, 0.8 mg/ml G418 and 0.3 mg/ml hygromycin (Gibco-BRL). Resistant clones were cloned by ring isolation after 4 weeks of selection.

Selection of src overexpressers

Lysates of each stable transfectant clone resistant to both neomycin and hygromycin were prepared for Western blot analysis as described (Lee *et al*., 1993; Ha-Lee *et al*., 2000). Lysates containing equal amounts of protein were resolved on 8% SDS-PAGE, the proteins were transferred to Immobilon-P (Millipore, Bedford, MA, USA) and the membranes were incubated with an anti-src monoclonal antibody (Oncogene, Cambridge, MA, USA) followed by rabbit anti-mouse IgG-peroxidase conjugate (PharMingen, San Diego, CA, USA). The src antibody-anti-mosue IgG complex was detected by using ECL reagent (Amersham, Piscataway, NJ, USA).

Results

A CD99 splice variant promotes motility of breast cancer cells

To investigate the functional effects of CD99 molecules on the metastatic phenotypes of cancer cells, several CD99 transfectant clones of MDA-MB-231 human breast adenocarcinoma cells lacking CD99 expression were generated. Those CD99 tranfectant clones stably overproduced either a major form (type I) or a splice variant (type II) of CD99 protein on cell surface. Among those CD99 transfectant clones, the CD99 type II-transfected clone displayed significantly increased invasiveness when compared with the control transfectant while the CD99 type I-transfected clone did not (Kim *et al*., 2000). Since invasiveness *in vivo* is closely reflect motility *in vitro*, the migrating ability of the CD99 transfectant clones was examined. As shown in Figure 1, the CD99 type II transfectant clone exhibited more than 3-fold higher migrating ability than the control transfectant, whereas no significant difference in cell motility was observed between the CD99 type I and control transfectant.

A FAK phosphatase inhibitor, phenylarsine oxide and a src kinase inhibitor, PP1 suppress cell motility of the CD99 type II transfectant

Both types of CD99 molecule have been shown to trigger intracellular signals leading to alterations in

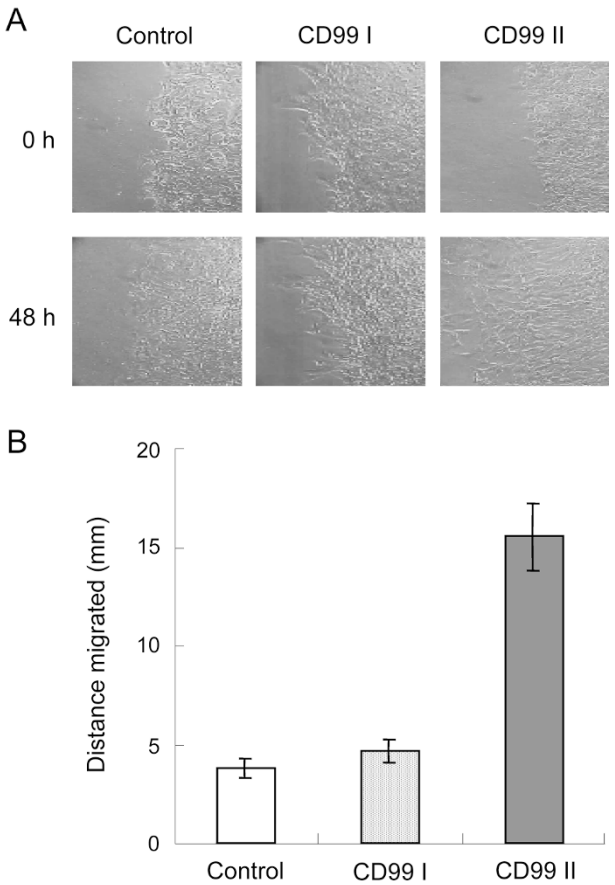

Figure 1. *In vitro* cell motility of the CD99 type I and type II transfectant clones. The CD99 type I- and type II-transfected MDA-MB-231 clones (5 x 10^5 cells) were seeded in each well of 24-well plates. When the cells were grown to confluent state, the cell layer was wounded with a plastic micropipette tip. (A) Photographs were taken at 48 h after wound was made, and the migrating distance of each cell was measured for cell motility. (B) Shown in a bar graph are mean ± SEM (n>30). The difference in motility between the control and CD99 splice variant transfectant cells was statistically significant (P<0.01, Student's *t*-test).

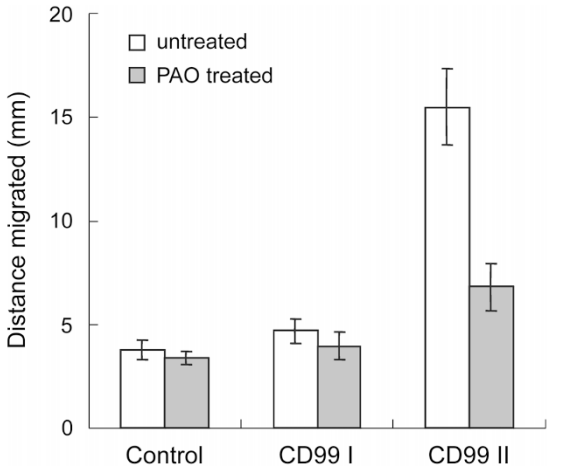

Figure 2. Cell motility of the CD99 type I and type II transfectant clones after treatment with an inhibitor of FAK phosphatase. The CD99 type I- and type II-transfected MDA-MB-231 cells were pretreated for 5 min with 5 μM phenylarsine oxide (PAO), an inhibitor of FAK phosphatase, or with vehicle control (DMSO) and then assayed for cell motility as in Figure 1. The difference in motility between the untreated and PAO-treated cells in the CD99 type II transfectant was statistically significant (P<0.01, Student's *t*-test).

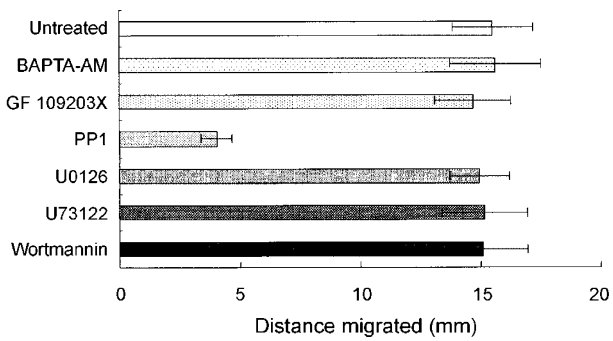

Figure 3. Cell motility of the CD99 type II transfectant in the presence of inhibitors of signal transduction mediators. The CD99 type II-transfected MDA-MB-231 cells were assayed for their migrating ability in the presence of BAPTA-AM (7.5 μM), GF109203X (1 μM), PP1 (1 μM), U0126 (1 μM), U73122 (1 μM) or wortmannin (50 nM), respectively. Data represent the mean ± SEM of triplicate determinations. The difference in motility between the untreated and PP1-treated cells was statistically significant (P<0.01, Student's *t*-test).

homotypic aggregation of various cell types. On the other hand, it would be difficult to accept that CD99 type II transfactant clones with far greater cell migrating activity would have the same cell signal path of the CD99 expressing cells. As an effort to explore signaling pathway(s) taken downstream of the CD99 type II for the induction of cell motility, we first examined migrating ability of the CD99 transfectant cells after treatment with phenylarsine oxide, an inhibitor of FAK phosphatase which plays an important role in signaling pathway of integrin-mediated cell migration. CD99 type II-induced cell motility was blocked for the most part by pretreatment with phenylarsine oxide (PAO) (Figure 2). Whereas, the intrinsic migratory capacity of MDA-MB-231 cells and CD99 type I transfectants were not much affected by PAO treatment. Such inhibition on motility of the CD99 type II-expressing cells by PAO suggests that focal adhesion kinase in associated with integrin may modulate the motility-promoting function of CD99 type II. Cell motility of the CD99 type II transfectant was also investigated in the presence of the other inhibitors for signal transduction mediators in a several-fold higher, but not lethal, dose than a dose of IC_{50}: BAPTA-AM, an intracellular calcium chelator; GF109203X, a PKC inhibitor; PP1, a src kinase inhibitor; U0126, an ERK kinase (MEK) inhibitor; U73122, a phospholipase C inhibitor; wortmannin, a PI-3 kinase inhibitor. As shown in Figure 3, the inhibitor of src kinase family, PP1 significantly suppressed cell motility of the CD99 type II transfectant. The PP1-treated cells were approximately 70% less motile than the untreated cells, whereas none of the other inhibitors tested affected cell motility of the

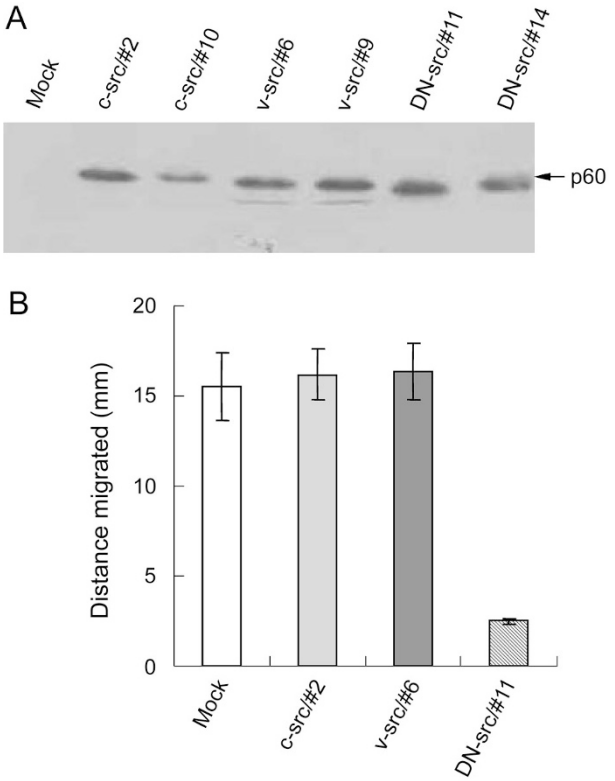

Figure 4. Cell motility of the CD99 type II transfectant after retransfection with the different types of src. (A) Src expression level of *src* transfectant clones. The CD99 type II-transfected MDA-MB-231 cells were retransfected with an expression construct encoding c-src, v-src or dominant-negative (DN) mutant src. Src protein level in each transfectant clone was examined by Western analysis using anti-src monoclonal antibody as described in Materials and Methods. (B) Cell motility of the *src* transfectant clones with CD99 type II expression. Three different types of *src*-transfected MDA-MB-231 clones that had been pretransfected with the CD99 type II were assayed for their migrating ability as in Figure 1. Data represent the mean ± SEM of triplicate determinations. The difference in motility between the mock and dominant-negative (DN) mutant *src* transfected cells was statistically significant ($P < 0.01$, Student's *t*-test).

CD99 type II transfectant. Because the inhibition effect of PP1 was not exerted at concentrations lower than 200 nM, it was likely that src kinase, the only kinase showing IC_{50} higher than 100 nM for PP1 among src family members, is functionally involved in signaling pathway(s) of the CD99 type II for the induction of motility of breast cancer cells.

Generation of different types of src-transfected stable clones with high src expression

To verify the signal-mediating role of src kinase in the CD99 type II signaling pathway to induce cell motility, we retransfected the CD99 type II-transfected MDA-MB-231 cells witd an expression construct enconding v-src, c-src or dominant-negative mutant src lacking kinase activity. Stable transfectant clones were screened for the overproduction of src protein on immunoblots using anti-src monoclonal antibody and identified several transfectant clones overexpressing src protein for each type of *src* construct (Figure 4A). Src protein levels in those *src* transfectant clones were far greater than an endogenous src protein level. And the exogenously introduced type of src kinase likely represents most of the biochemical activity exerted by src kinase in those double transfectant cells overexpressing both CD99 type II and one of the src protein types.

Overexpression of mutant src protein lacking kinase activity inhibits cell motility of the CD99 type II transfectant

Analysis of cell motility of the double transfected cells with CD99 type II and different types of *src* transfection is shown in Figure 4B. The overexpression of v-src and c-src protein does not significantly affect motility of the CD99 type II-transfected MDA-MB-231 cells. However, the dominant-negative mutant *src*-transfected clones displayed 1/5 level of cell motility of the mock transfectant having endogenous src expression only, indicating that overexpression of kinase-negative src mutant protein interferes the promoting effect of CD99 type II on motility of MDA-MB-231 cells. This result strongly suggests that the CD99 type II induce motility of human breast cancer cells through src kinase-dependent pathway.

Discussion

Several studies have demonstrated the role of the major form of CD99 (type I) as a signal transducer for the induction of homotypic aggregation of immune cells, although the ligand(s) for CD99 is not yet known (Bernard *et al*., 1995; Hahn *et al*., 1997). Cross-linking of CD99 type I with an anti-CD99 monoclonal antibody (mAb) up-regulated surface LFA-1 α_L integrin expression of IM-9 B and Jurkat T cells, together with induction of hymotypic cell aggregation (Hahn *et al*., 1997). Stimulation of CD99 type I with anti-CD99 mAb enhanced the expression of T cell activation markers, CD25, CD69 and CD40L on anti-CD3-activating T cells, along with elevation of intracellular Ca^{2+} and the tyrosine phosphorylation of cellular proteins (Waclavicek *et al*., 1998; Wingett *et al*., 1999). Induction of Jurkat homotypic aggregation by anti-CD99 mAb was blocked by the protein kinase C inhibitor, sphingosine and the protein tyrosine kinase inhibitor, genistein (Kasinrerk *et al*., 2000). Treatment of Jurkat cells with anti-CD99 mAb led to differential activation of three mitogen-activated protein kinase (MAPK) members, ERK, JNK and p38 MAPK (Hahn *et al*., 2000). The signal-transducing role of CD99 type I for the induction of homotypic cell aggregation was not observed only in hematopoitic

cells, but also in breast cancer cells of epithelial origin; that is, cross-linking of CD99 type I with anti-CD99 mAb induced the homophilic cell adhesion of MDA-MB-231 human breast cancer cells (Kim *et al*., 2000). On the contrary, we also found that activation of a CD99 splice variant (type II) with anti-CD99 mAb inhibits homotypic aggregation of the CD99 type II-transfected MDA-MB-231 cells. Since the sole activation of CD99 type II was able to suppress the homotypic aggregation of MDA-MB-231 breast cancer cells devoid of endogenous CD99 type I expression, the CD99 splice variant might be able to trigger its own signal transduction pathway, instead of simply interfering a signaling pathway of the major CD99 form.

We have previously found that expression of the CD99 spice variant increases invasive ability of MDA-MB-231 breast cancer cells (Kim *et al*., 2000). When the CD99 type II transfectant cells were assayed for cell motility, the expression of the CD99 type II obviously increased the migrating ability of MDA-MB-231 cells (Figure 1). Since an alteration in physiological constraints that enable typically non-motile epithelial cells to migrate is a hallmark of cancer invasion and metastasis, the increase in invasiveness of breast cancer cells by the CD99 type II expression seems to be mostly due to the motility-promoting function of CD99 type II. To reveal the role of CD99 type II as a signal transducer, we have explored signal transduction pathway(s) taken downstream of the CD99 type II for the induction of motility of MDA-MB-231 cells. The data in the present study show that a FAK phosphatase inhibitor, phehylarsine oxide and a src kinase inhibitor, PP1 significantly blocked cell motility induced by the CD99 type II expression while inhibitors of other major signaling mediators did not (Figures 2 and 3). We also found that overexpression of src mutant lacking kinase activity suppresses the CD99 type II-induced cell motility to a great extent (Figure 4). It thus appears that src kinase indeed mediates the functional role of CD99 splice variant in the regulation of motility of human breast cancer cells, along with focal adhesion kinase.

Cell migration is a muti-step process that requires repeated adhesion to and detachment from extracellular matrix (ECM). These events are largely mediated by integrins, which upon engagement with components of the ECM reorganize to form adhesion complexes termed focal adhesion complexes. The integrin clustering results in increased phosphorylation of a protein tyrosine kinase localized at the focal adhesion complexes, focal adhesion kinase (FAK) (Guan and Shalloway, 1992; Kornberg *et al*., 1992; Lipfert *et al*., 1992; Miyamoto *et al*., 1995). Upon integrin engagement, FAK becomes phosphorylated on tyrosine-397, creating a high affinity-binding site for src and src family kinases (Schaller *et al*., 1994). The FAK/Src complex, in turn mediates phosphorylation of several associated focal adhesion proteins, in addition to phosphorylation of additional sites on FAK by src kinase (Calalb *et al*., 1995; Polte and Hanks, 1995; Schaller and Parsons, 1995; Calalb *et al*., 1996). Although several substrates of the FAK/src bipartite kinase complex have been recently identified as regulators of migration (Cary *et al*., 1998; Klemke *et al*., 1998; Petit *et al*., 2000), the mechanisms whereby FAK/src signaling pathways regulate migration are largely unknown.

Several studies have reported elevated expression and activity of FAK and src kinase in advanced progression stages of several types of human cancer including colon, thyroid, prostate and ovarian cancers (Talamonti *et al*., 1993; Owens *et al*., 1996; Tremblay *et al*., 1996; Mao *et al*., 1997; Judson *et al*., 1999). It was recently reported that overexpression of the dominant-negative mutant types of FAK and src kinase suppresses motility and invasiveness of human carcinoma cells (Huak *et al*., 2001; Sakamoto *et al*., 2001). Slack *et al*. (2001) also showed a correlation between alterations in FAK/src signaling pathway and increased migratory capacity of prostate cancer cells. The involvement of FAK and src in the regulation of motility and invasion was also revealed in breast cancer cells by the following results from several groups: (1) FAK expression level is strongly associated with invasion and metastasis of breast cancer (Weiner *et al*., 1993; Cance *et al*., 2000); (2) FAK can modulate an inhibitory effect of sphingosine 1-phosphate on motility of MDA-MB-231 breast cancer cells (Wang *et al*., 1999); (3) Src kinase is required for hepatocyte growth factor-induced motility of mouse mammary carcinoma cells (Rahimi *et al*., 1998). Therefore, it is likely that any signaling pathway leading to an increase in expression and activity of focal adhesion kinase and src kinase could promote motility of breast cancer cells. Although it is uncertain whether the CD99 type II expression correlates with a malignant progression of breast cancer, the data in this study suggest that the CD99 slice variant is one of the molecules which can activate focal adhesion kinase and src kinase by triggering its own signal transduction pathway for the induction of motility and invasiveness of human breast cancer cells.

Acknowledgements

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