

# CD44 crosslinking-mediated matrix metalloproteinase-9 relocation in breast tumor cells leads to enhanced metastasis

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Received May 21, 2007; Accepted July 17, 2007

**Abstract.** CD44 plays a major role in multiple physiological processes, including cell-cell adhesion, cell-substrate interaction, lymphocyte homing, and tumor metastasis. It has been reported that highly expressed CD44 in certain types of tumors is associated with the hematogenic spread of tumor cells. The ability of CD44 to bind hyaluronan has been shown to correlate with tumor cell invasiveness, and it is likely that this ability may enhance tumor cell migration at several points during metastasis. However, the mechanism as to how CD44 stimulates metastasis remains unknown. The human breast tumor cell line, MDA-MB-435s, was used to investigate the effect of antibody-mediated CD44 crosslinking on the cellular level and localization of matrix metalloproteinase-9 (MMP-9). Confocal microscopy and immunocytochemical analyses were performed to demonstrate colocalization of CD44 and MMP-9 after CD44 crosslinking. Furthermore, the CD44-MMP-9 complex was purified by immunoprecipitation. G8 myoblast monolayers were employed to evaluate the invasiveness of human breast tumor cells after CD44 crosslinking in the presence or absence of protease inhibitors. CD44 crosslinking augmented the level of MMP-9 in the membrane of human breast tumor cells and clustering of CD44 serves as an MMP-9 docking molecule allowing MMP-9 to retain its concentrated proteolytic activity on the cell surface. Furthermore, crosslinking of CD44 enhances the ability of breast tumor cells to invade G8 myoblast monolayers and migrate through the basal membranes which was inhibited in the presence of anti-MMP-9 antibody or the MMP inhibitors GM6001 or 1,10-phenanthroline. This study demonstrates for

the first time that CD44 crosslinking leads to an enhanced level and relocation of MMP-9 in human breast tumor cells accompanied by increased tumor invasion and metastasis.

## Introduction

Tumor metastasis is the process by which individual or a subset of cancer cells disseminate from a primary tumor to distant secondary tissues involving many factors such as interactions with the extracellular matrix, molecular signaling, and hereditary influences (1,2). Dalchau *et al* (3) discovered that T lymphocytes contain CD44 antigens that are consistently overproduced by various types of tumor cells. CD44 is encoded by the 50-Kb CD44 gene that includes 20 exons (4). Exon 6-15 (V<sub>1</sub>-V<sub>10</sub>) located in the variable region is responsible for the generation of isoforms through extensive alternative splicing (4-6). The CD44 standard form (CD44s, 85-95 kDa) in which variant exons V<sub>1</sub>-V<sub>10</sub> are not expressed is the most common and smallest CD44 isoform. CD44 is involved in the direction of cell-to-cell and cell-to-intercellular matrix interactions (7-9) and can be activated using cytokines or antibodies which increase the affinity (10-12) for its main ligand hyaluronan (13,14). Interestingly, the use of antibodies to crosslink CD44 strengthens the CD44-hyaluran binding (15). Binding of CD44 with hyaluronan affects cell adhesion to extracellular matrix components which is implicated in subsequent cell migration and metastasis (16).

Various types of highly developed tumors exhibit substantial levels of CD44 which suggested that CD44 is directly related to tumor invasion and metastasis (17,18). Antibodies- and hyaluronan-fragment-mediated CD44 crosslinking induces the production of adhesion molecules, including lymphocyte function-associated antigen (LFA-1), very late antigen-4 (VLA-4), VCAM-1, and ICAM-1 (19-21). Bourguignon *et al* (22) discovered that the ligation of hyaluronan and CD44v3 enhances the interaction between CD44v3 and T lymphoma invasion and metastasis 1 (Tiam1) proteins, activates Rac 1 signaling, and promotes breast tumor cell migration. In addition, c-Src kinase, Ras, protein kinase C, and IκB kinase 1 and 2 were found to be participants in the signaling pathways of hyaluronan stimulation (22,23), illustrating that CD44 acts as a signaling molecule. In fact, hyaluronan receptor-CD44

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*Key words:* MMP-9, CD44, metastasis, breast tumor cells

complexes play a crucial role in adhesion and the growth and spread of various tumor cells (8,24-26).

Matrix metalloproteinases (MMPs) have been considered as crucial molecules aiding tumor cells during metastasis and are members of a group of 28 proteases located within the extracellular matrix and involved in its degradation. Because of the extracellular matrix component-degrading capacity of MMPs, they participate in tumor cell invasion and migration (27). MMP-9 (gelatinase B, 92 kDa; 28) belongs to the gelatinase class of MMPs and is characterized by the presence of three repeating fibronectin-type II domains near the catalytic domain that enhances its interaction with collagens and gelatins (29,30) and plays an important role in tumor cell invasion and migration (2).

The interrelatedness of CD44 activation and MMP-9 gene expression and protein levels, and its effect on metastasis has been widely reported for various types of tumor cells. Yu and Stamenkovic (31) demonstrated that CD44 associates with a proteolytic form of MMP-9 on the surface of mouse mammary carcinoma and human melanoma cells forming a complex that promotes tumor cell invasion of G8 myoblast monolayers. Interestingly, hyaluronan oligosaccharides that bind the CD44 receptors induce MMP-9 expression in murine 3LL tumor cells via receptor, but not CD44-receptor, mediated mechanism (32). CD44 and hyaluronan modulated leukocyte migration in a mouse zymosan air-pouch inflammation model also involved MMP-9 (33) and CD44 activation also led to an increased invasion and expression of MMP-9 in a human colorectal cell line (34). Recently, it has been shown that in a colon cancer cell line, dentin matrix protein 1 was involved in bridging MMP-9 to cell surface receptors, including CD44 (35). Those studies have important pharmacological applications as demonstrated by Annabi *et al.* (36) who developed the synthetic peptide, PCK3145, that inhibits MMP-9 secretion and triggers CD44 cell surface shedding in a fibrosarcoma cell line, and thus exhibits an antagonistic effect on tumor cell metastasis.

Here, we used a human breast tumor cell line exhibiting large quantities of CD44 receptor and evaluated the effect of CD44 crosslinking on the intra- and intercellular level of MMP-9. In addition, *in vitro* assays were conducted to determine whether MMP-9 is involved in tumor cell migration and metastasis after CD44 crosslinking.

## Materials and methods

**Cell cultures.** Human breast tumor cell line (MDA-MB-435s) and mouse myoblast cell line (G8) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). MDA-MB-435s cells were cultivated in Leibovitz's L-15 medium supplemented with 15% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 10  $\mu$ g/ml insulin, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells of the mouse myoblast cell line (G8) were grown on collagen-coated dishes containing DMEM, supplemented with 10% FBS, 10% horse serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

**Crosslinking of CD44.** Cells were cultured to subconfluence and then incubated with mouse anti-CD44 antibody (10  $\mu$ g/ml) (Newmarker, Fremont, CA, USA) for 1 h at 37°C. After

washing the cells three times, 1  $\mu$ g/ml of goat anti-mouse IgG-Fc (Bethyl, Montgomery, TX, USA) was added as the second antibody for CD44 crosslinking.

**Flow cytometric analysis.** Suspended breast tumor cells ( $5 \times 10^6$  cells/ml), with or without CD44 crosslinking, were incubated for 45 min at 4°C with 150  $\mu$ l of appropriate FITC-conjugated antibody solution (10  $\mu$ g/ml), washed with phosphate-buffered saline (PBS), spun down, and then fixed in 1.5 ml of 4% paraformaldehyde. Negative control samples were incubated with PBS in place of the primary antibody. A Becton-Dickinson FACSCalibur Analytic Flow Cytometer (Palo Alto, CA, USA) was used to analyze antibody binding.

**Immunocytochemistry of breast tumor cells.** Staining was performed on fixed, non-permeabilized monolayers of breast tumor cells grown on glass tissue culture slides. Cells were washed with PBS and fixed with 0.05% glutaraldehyde for 30 min at 37°C. Monolayers were incubated with 5% non-fat milk in PBS for 25 min at room temperature to block non-specific binding. Mouse anti-CD44 mAb (1:100) and rabbit anti-MMP-9 pAb (1:100) were added and the slides were incubated for 75 min at room temperature. The secondary antibodies were rhodamine-conjugated donkey anti-mouse Ab (1:100) or FITC-conjugated goat anti-rabbit Ab (1:100). Cells were incubated with secondary antibody for 60 min at room temperature and then mounted with mounting medium (Vector, North Hollywood, CA, USA). Observations were performed with a confocal laser scanning microscope (Leica, Bensheim, Germany) using a 100x/1.30 oil immersion objective and an appropriate filter.

**Immunoblotting and immunoprecipitation techniques.** For immunoblotting on polyvinylidene difluoride (PVDF) filter membranes, cells were pooled and briefly rinsed with PBS. The cell pellets were lysed in 1 ml of lysis buffer consisting of PBS, 1% sodium dodecyl sulfate (SDS) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml soybean trypsin inhibitor, and 0.5 mM dithiothreitol. The supernatant was removed after centrifugation for 20 min at 2,650 rpm at 4°C, and then ultracentrifuged at 55,000 rpm for 1 h at 4°C. The supernatant contains the cytosolic fraction and the pellet is the membrane fraction. The media were also collected, concentrated by Centricon YM-30, and lyophilized. The samples were analyzed by SDS-PAGE using a 10% polyacrylamide gel. Separated polypeptides were then transferred onto PVDF membranes. After blocking non-specific sites with 5% non-fat milk, the PVDF membranes were incubated with each of the specific immunoreagents [e.g., rat anti-CD44 Ab (5  $\mu$ g/ml), and rabbit anti-MMP-9 Ab (5  $\mu$ g/ml)], followed by an incubation with horseradish peroxidase-labeled immunoreagents (e.g., goat anti-rat Ab, goat anti-rabbit Ab, or goat anti-mouse Ab). The blots were developed using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

For analyzing the complex formation between CD44 and MMP-9, MDA-MB-435s cells (with or without CD44 crosslinking for 12 h) were solubilized by lysis buffer and then ultracentrifuged to separate cytosolic and membranous

fractions. The media were also collected and either immunoprecipitated with mouse anti-CD44 mAb (2  $\mu$ g of primary antibody per 1 mg of protein sample; 0.6  $\mu$ g of primary antibody per 1 ml medium) overnight at 4°C, and then immunoblotted with anti-MMP-9, or immunoprecipitated with MMP-9 antibody before being immunoblotted with anti-CD44. Proteins were resolved by 12% SDS-PAGE, transferred to PVDF and subsequently probed with mAbs as indicated. Immunoreactive polypeptides were visualized using horseradish peroxidase-labeled secondary antibodies and the ECL detection system.

**Gelatin zymography.** Gelatin zymography was carried out as described by Shi *et al* (37) with some modifications. Briefly, gelatin (1.5 mg/ml) as a substrate was copolymerized with regular 10% SDS-polyacrylamide gel. Electrophoresis was performed at a constant current of 15 mA. After electrophoresis, the gel was washed with 2.5% Triton X-100 to remove the SDS, and incubated in buffer (50 mM Tris-Cl, pH 8.0, 50 mM NaCl, and 10 mM CaCl<sub>2</sub>) in a 37°C water bath for 24 h to degrade the gelatin. The gel was then removed from the zymogram incubation buffer and stained in ethanol-based Coomassie Blue solution for 1 h to observe the lysis band.

**Invasion assay.** The assay used here was described by Yu *et al* (38). Briefly, G8 cells were grown in collagen coated 6-well plates until the monolayers were confluent, and then fixed with 20% dimethyl sulfoxide in methanol for 1 h. After washing with PBS, the MDA-MB-435s cells [ $2 \times 10^5$  cells with or without CD44 crosslinking for 12 h or with or without pretreatment with rabbit anti-MMP-9 polyclonal Ab (10  $\mu$ g/ml), MMP inhibitor GM6001 (Chemicon, Temecula, CA) (0.2 nM), or MMP inhibitor 1,10-phenanthroline (Sigma, St. Louis, MO) (2.5 mM) for 1 h before CD44 crosslinking] were added to the fixed cell layer and incubated in a CO<sub>2</sub> incubator for 6 days, and then observed with a phase contrast microscope.

## Results

**MMP-9 level and zymogram detection of active MMP-9 in breast tumor cells after CD44 crosslinking.** To evaluate the effect of CD44 crosslinking on the cellular level of MMP-9 as a function of time, human breast tumor cells (MDA-MB435s) were incubated with mouse anti-CD44 mAb for 1 h and goat anti-mouse IgG Fc Ab for 6, 9, 12, 24 h to crosslink the CD44 receptors. After goat anti-mouse IgG Fc Ab addition, the protease-containing subcellular fractions and serum-free medium were collected and subjected to Western blot analyses. Prior to CD44 crosslinking, MMP-9 (molecular weight ca. 92 kDa) is detected in the membrane of MDA-MB-345s cells (Fig. 1A). A gradual increase in the CD44 receptor crosslinking time from 1 to 24 h resulted in an increased amount of MMP-9 in the membrane fraction (Fig. 1A) reaching a maximum after 12 h of CD44 receptor crosslinking to decrease by 24 h of CD44 crosslinking (Fig. 1A). Western blot analyses of the lyophilized serum-free medium fraction demonstrated that for all the time points tested, the level of the MMP-9 was highest at 24 h of crosslinking (Fig. 1B).

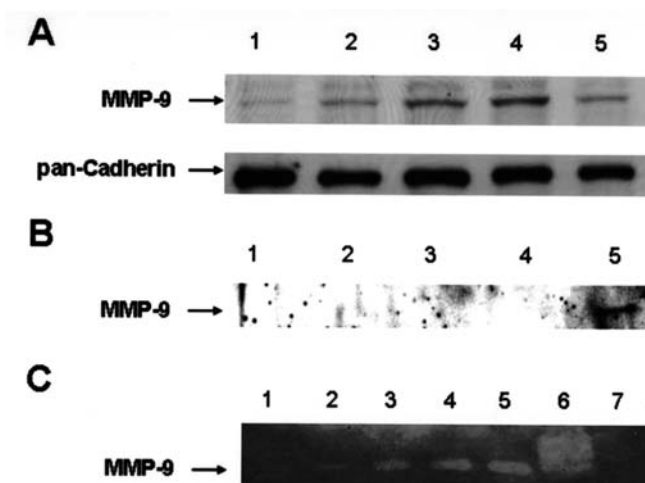


Figure 1. Evaluation of the MMP-9 levels in the membrane of MDA-MB-435s human breast tumor cells after CD44 crosslinking for 1-24 h. The crosslinking of CD44 receptors in MDA-MB-435s cells was performed for 6, 9, 12, or 24 h and the levels of MMP-9 in (A) cell membrane and (B) serum-free medium were detected by Western blotting. Lane 1, control group without CD44 crosslinking; lanes 2-5, CD44 crosslinking for 6, 9, 12, and 24 h, respectively. The relative intensity of the bands in lanes 2-5 was 2.8, 4.1, 5.6, and 3.3, respectively (the intensity of the control band in lane 1 was set at 1). (C) CD44 receptors of MDA-MB-435s cells were stimulated for 3-36 h. Serum-free medium was collected and zymograms were used to assess MMP-9 activity after 3, 6, 9, 12, 24, and 36 h CD44 crosslinking (lanes 1-6, respectively). Lane 7 contained the control when no crosslinking was applied.

Furthermore, gelatin zymogram analyses were performed after 3, 6, 9, 12, 24, and 36 h of CD44 crosslinking; samples of serum-free medium were collected along with samples from the corresponding control (Fig. 1C). Through the Coomassie blue-stained background proteins, the MMP-9 lysis band was distinguishable at 24 h of CD44 receptor crosslinking. The MMP-9 level was highest at 24 h of CD44 crosslinking (Fig. 1C).

**CD44 crosslinking enhances the level of MMP-9 at the breast tumor cell surface.** After 12 h of CD44 crosslinking, MDA-MB-435s cells were trypsinized and the resulting suspension was treated with rabbit anti-MMP-9 polyclonal antibody, followed by FITC-conjugated goat anti-rabbit Ab, and analyzed by flow cytometry. No MMP-9 was detected in control MDA-MB-435s cells that were not subject to CD44 crosslinking nor in negative control cells that were not subject to CD44 crosslinking and that were not incubated with secondary FITC-conjugated goat anti-rabbit Ab (Fig. 2A). In contrast, a large amount of MMP-9 was found on the surface of cells after 12 h of CD44 crosslinking (Fig. 2B).

**Colocalization of MMP-9 and CD44 after 12 h CD44 crosslinking.** To investigate the possible colocalization of MMP-9 after 12 h of CD44 receptor crosslinking, MDA-MB-435s cells were fixed, blocked, and incubated with mouse anti-CD44 mAb and rabbit anti-MMP-9 pAb at room temperature. Control cells were treated with rhodamine-conjugated donkey anti-mouse Ab, while the cells with crosslinked CD44 were treated with FITC-conjugated goat anti-rabbit Ab and observed using a confocal microscope. Cells that did not



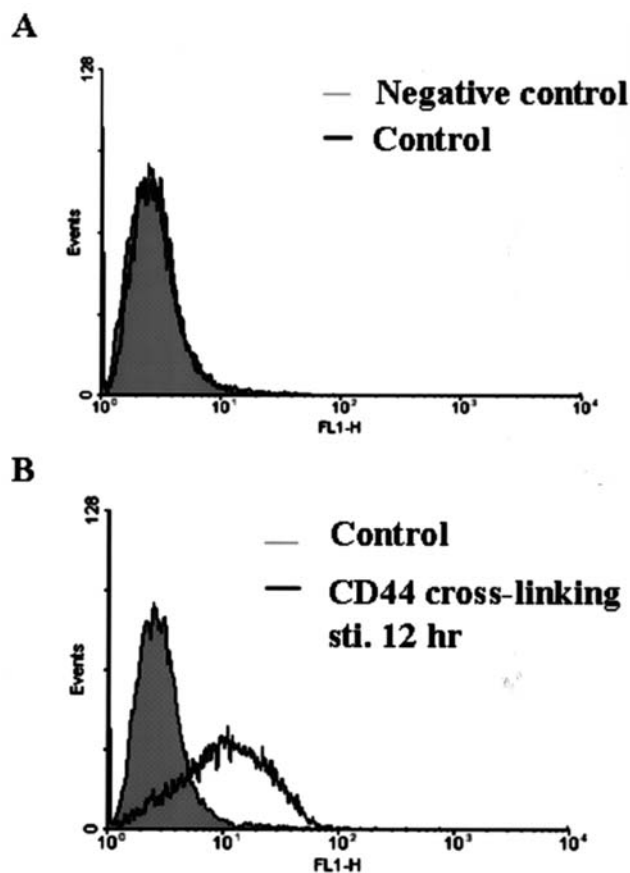


Figure 2. Evaluation of the MMP-9 level on cell membranes of MDA-MB-435s cells after 12 h CD44 crosslinking by flow cytometry. Comparison between (A) the control cells without CD44 crosslinking and the negative control in which the second antibody was omitted; and (B) the control cells and stimulated cells after 12 h of CD44 crosslinking.

undergo CD44 crosslinking were found to have CD44 receptors on their membranes (Fig. 3B) and MMP-9 in their cytosols (Fig. 3A) as confirmed by the composite image (Fig. 1C). Cells with CD44 receptor crosslinking were found to have caps of CD44 (Fig. 3E) and large amounts of MMP-9 on their membranes (Fig. 3D) which was confirmed by the composite image (Fig. 3F).

*Immunoprecipitation of the CD44-MMP-9 complex after CD44 crosslinking.* Subcellular fractions and culture medium of MDA-MB-435s cells were collected after 12 h of CD44 crosslinking and the CD44 protein complexes were immunoprecipitated with mouse anti-CD44 mAb coupled to protein A-agarose beads. Proteins binding to CD44 receptors were purified and analyzed by Western blotting using rabbit anti-MMP-9 to observe the interaction between CD44 receptor and MMP-9 showed that MMP-9 is on the cell membranes of breast tumor cells after CD44 crosslinking for 12 h. This biochemical experiment supports the colocalization study and suggests that the 92-kDa MMP-9 and CD44 receptor appear as a complex on the cell membrane (Fig. 3G).

*Effect of cycloheximide on induction of MMP-9 expression by CD44 crosslinking.* MDA-MD-435s cells were treated with 20  $\mu\text{g/ml}$  of the protein synthesis inhibitor cycloheximide

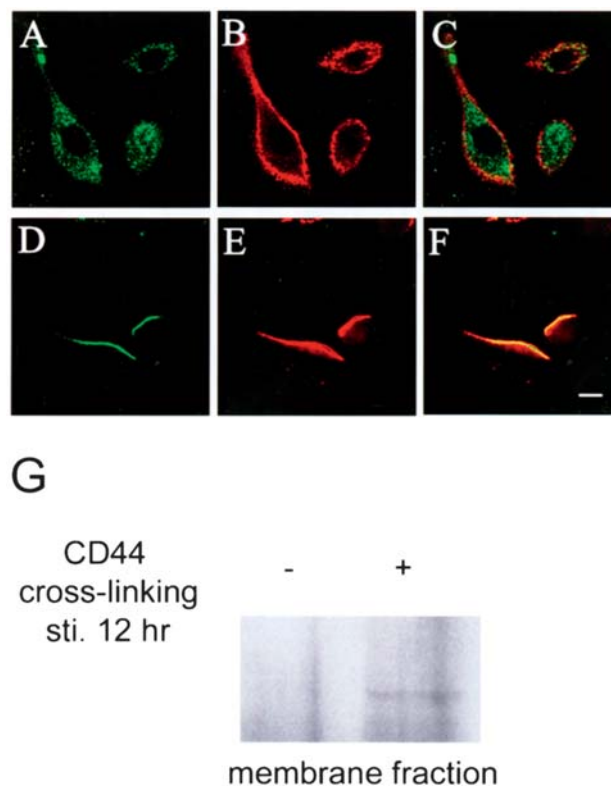


Figure 3. Localization of CD44 and MMP-9 in MDA-MB-435s cells after CD44 crosslinking for 12 h. Double immunofluorescent staining of (A) MMP-9 and (B) CD44 in control cells, and (D) MMP-9 and (E) CD44 in cells after CD44 crosslinking for 12 h. (C) is a composite image of (A) and (B), while (F) is a composite image of (D) and (E). (Scale bar, 10  $\mu\text{m}$ .) (G) After 12 h of CD44 crosslinking, mouse anti-CD44 mAb and protein A agarose beads were added to immunoprecipitate CD44, and the expression of MMP-9 was found to be colocalized with CD44 on cell membranes.

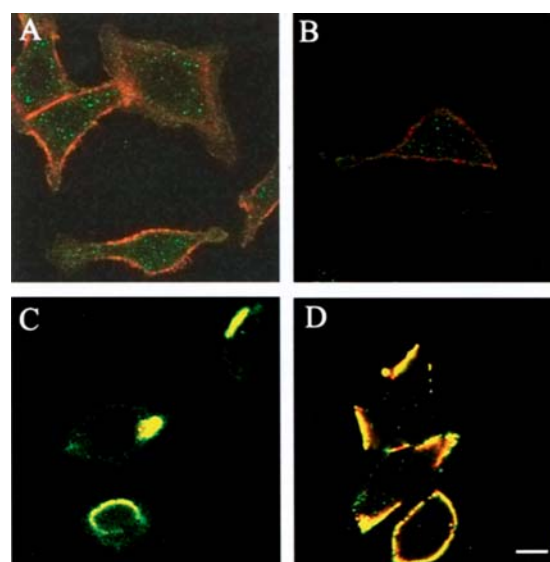


Figure 4. Localization of MMP-9 in MDA-MB-435s cells after treatment with cycloheximide followed by CD44 crosslinking for 12 h. Double immunofluorescent staining to observe the localization of CD44 receptor (red) and MMP-9 (green) in MDA-MB-435s cells. A, control; B, cells treated with cycloheximide; C, CD44 crosslinking without the addition of cycloheximide; D, cells first treated with cycloheximide followed by 12 h CD44 crosslinking. Scale bar, 10  $\mu\text{m}$ .

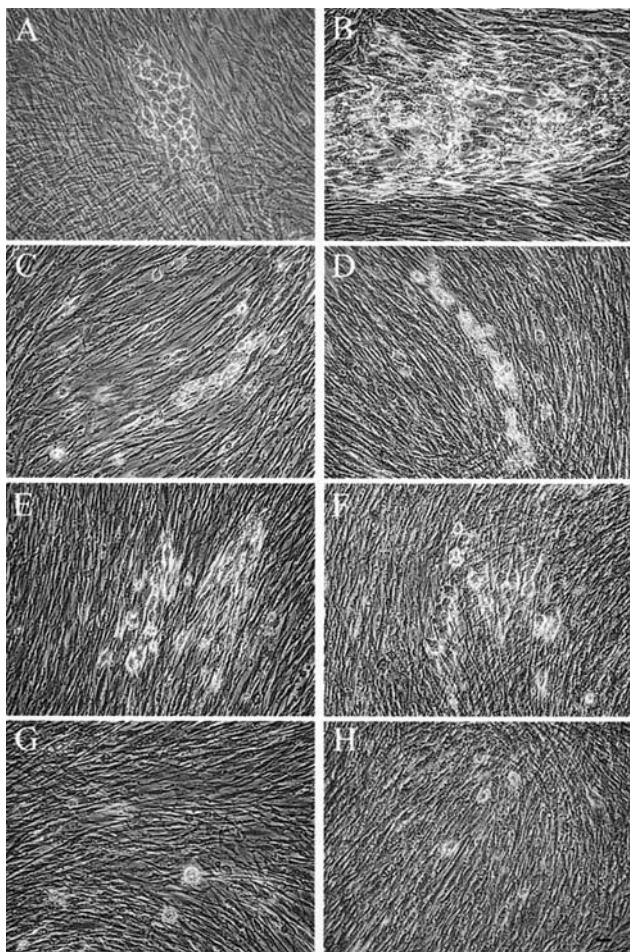


Figure 5. Invasion of G8 myoblast monolayers by breast tumor cells. Tumor cell islets (arrow, breast tumor cells) were observed to invade the monolayer 6 days after being seeded with breast tumor cells. Before seeding dimethyl sulfoxide-fixed G8 monolayers with MDA-MB-435s cells, the latter were or were not subjected to CD44 crosslinking at 37°C for 12 h. A, Control, breast tumor cells; B, stimulated by CD44 crosslinking for 12 h; C, treated with rabbit anti-MMP-9 pAb (10 µg/ml); D, pretreated with rabbit anti-MMP-9 pAb (10 µg/ml), before CD44 crosslinking; E, treated with MMP inhibitor, GM6001 (0.2 nM); F, treated with MMP inhibitor, GM6001 (0.2 nM), before CD44 crosslinking; G, treated with 1,10-phenanthroline (2.5 mM); and H, treated with for 1,10-phenanthroline before CD44 crosslinking. Scale bar, 10 µm.

for 1 h, subject to 12 h of CD44 crosslinking, fixed, blocked, and treated with mouse anti-CD44 mAb and goat anti-MMP-9 pAb for 1 h at room temperature. The control cells (without crosslinking) were treated with rhodamine-conjugated donkey anti-mouse Ab for 1 h at room temperature. Both the CD44-crosslinked MDA-MD-435s cells and control cells were incubated with FITC-conjugated goat anti-rabbit Ab at room temperature for 1 h. The intracellular localization of MMP-9 and CD44 was evaluated by confocal microscopy.

The results showed that prior to CD44 crosslinking, CD44 is found on MDA-MB-435s cell membranes while MMP-9 is found inside cells (Fig. 4A); treatment with cycloheximide did not alter this distribution (Fig. 4B). In addition, after 12 h of CD44 crosslinking, CD44 receptor and MMP-9 were found to be colocalized on cell membranes (Fig. 4C) and this distribution was not affected by cycloheximide (Fig. 4D). These observations suggest that CD44 crosslinking-mediated effects on MMP-9 do not require protein synthesis.

*Effect of CD44 crosslinking on breast tumor cell invasion of adjacent myoblast monolayers.* After addition of protease inhibitor [rabbit anti-MMP-9 polyclonal Ab (10 µg/ml), MMP inhibitor GM6001 (0.2 nM) or MMP inhibitor 1,10-phenanthroline (2.5 mM)] to breast tumor cells without or with crosslinking for 12 h,  $2.5 \times 10^2$  cells were collected and seeded on a G8 myoblast monolayer. Taking advantage of G8 myoblast's ability to form large amounts of substrate, we observed that the tumor cells were capable of invading adjacent healthy tissues, forming a colony with a complete and more rounded periphery (Fig. 5A). After 12 h of CD44 crosslinking, the ability of the tumor cells to invade adjacent tissues was noticeably enhanced, i.e., the colonies formed were larger, irregular, and had incomplete borders (Fig. 5B). This finding suggests that crosslinking of CD44 receptors stimulates tumor cells to invade adjacent tissues. The importance of matrix metalloprotease is clearly shown by the large amount of MMP-9 found on cell membranes after CD44 crosslinking. In this experiment, breast tumor cells were first incubated in rabbit anti-MMP-9 pAb and then subjected to crosslinking. It was observed that the invasive ability of the breast tumor cells was initially enhanced but subsequently became significantly repressed (Fig. 5C and D). In addition, the invasion ability of MDA-MB-435s cells treated with GM6001 and then subjected to CD44 crosslinking was reduced to the point where only small colonies could be formed (Fig. 5E and F). Similarly, the invasive ability of MDA-MB-435s cells treated with 1,10-phenanthroline before CD44 crosslinking was entirely eliminated (Fig. 5G and H). These findings suggest that breast tumor cell invasion involves a process of MMP-9 redistribution in response to CD44 receptor crosslinking.

## Discussion

In this study, we demonstrate for the first time in breast tumor cells that: i) crosslinking of CD44 on human breast tumor MDA-MB-435s cells enhances the relocalization of MMP-9 on cell membrane and ii) an increase of the MMP-9 level and its relocation after CD44 crosslinking stimulates breast tumor cell invasion of adjacent normal tissues and metastasis.

CD44 receptors are multifunctional involved in direct cell-cell and cell-substrate interactions, and serve as the main hyaluronan receptor on cell membranes. The stimulation of CD44 receptors by hyaluronan on human alveolar macrophages is molecular weight-dependent. Hyaluronan with smaller molecular weights was found to induce the expression of chemokine gene on human alveolar macrophages (39) and the expression of MMP-encoding genes in murine 3LL tumor cells (32). In other experiments, hyaluronan with different molecular weights was shown to stimulate nuclear factor-κB- and activating protein-1-mediated expression of VCAM-1 and ICAM-1 in mouse murine kidney tubular epithelial cells (19). We discovered that CD44 crosslinking induces integrin-mediated adhesion and transendothelial migration (40). These observations suggest that the signaling function of hyaluronan likely depends on its molecular weight.

Besides the ligand-mediated activation of CD44, it is also possible to activate CD44 by antibody-mediated crosslinking.



Indeed, Galandrini *et al* (41) used antibodies to stimulate the CD44 receptors on the human NK cell and enhanced cytotoxicity and Fujii *et al* (20) used antibodies for CD44 crosslinking in rheumatoid synovial fibroblasts to upregulate VCAM-1 expression. Here, we applied mouse anti-human CD44 mAb and goat anti-mouse IgG Fc antibody for CD44 crosslinking. Crosslinking acts as a CD44 receptor stimulant, thereby increasing the efficiency of signaling.

In breast cancer patients, tumor cells often metastasize to the bone. Saad *et al* (42) studied *in vitro* cocultures to determine the relationship between tumor cells and bone marrow fibroblasts. The tumor cells can use MMPs to enter capillaries, invade the tissue, and increase the area of the tumor, rendering cancer treatment more difficult (27). MMP-1 and MMP-2 were found to be present in substantial amounts in the medium which may possibly be perceived as a signal for breast tumor cells to penetrate the cellular layer and enter the bone marrow (42). In addition, evidence obtained from *in vitro* and *in vivo* experiments suggests that CD44 plays an important role in the preferential spread of metastatic breast cancer cells to the skeleton (43).

When breast tumor cells undergo CD44 crosslinking, the time of CD44 crosslinking is correlated with an increase of the cellular level of MMP-9 which peaked after 12 h of CD44 crosslinking, whereas of all time-points tested, the accumulation of MMP-9 in serum-free medium was highest at 24 h of stimulation. After CD44 crosslinking, large amounts of MMP-9 accumulate in the medium and cell membrane. Zymogram analyses showed that after 24 h of CD44 crosslinking, more of the more-active form of MMP-9 can be found, which corresponds to our Western blot results. Seftor *et al* (44) discovered that stimulating human melanoma  $\alpha\beta 3$  integrin increased the expression of MMP-2 and CD44 receptor stimulation in human melanoma increased expression of MMP-2 (45). These studies showed that stimulating adhesion molecules induces the production of proteases and is involved in the process of tumor cell invasion and migration. Thus, the biological effect of CD44 crosslinking varies with cell type and duration of stimulation.

Remarkably, no MMP-9 was detectable by flow cytometry in tumor cells that did not undergo CD44 crosslinking which may be due to its very low presence on cell surfaces. Under normal circumstances, a small amount of MMP-9 is detectable by Western blotting; this may be because the protease is collected from the entire cell, thus magnifying the signal. After 12 h of CD44 crosslinking, MMP-9 was found to be highly expressed on the cell membrane, thus proving that signaling occurs after CD44 receptor stimulation and induces MMP-9 expression on cell membranes.

It is noteworthy that CD44 receptors and MMP-9 clustered and colocalized. What is the biological consequence of this phenomenon in respect to the development of breast tumor cells? In the MMP-9 family, other than membrane MMPs that anchor to cell surfaces, most MMPs are secreted (28). Brooks *et al* (46) discovered that  $\alpha\beta 3$  integrin induces MMP-2 to localize on cell surfaces and overproduction of CD44 receptor in mouse mammary carcinoma and human melanoma cells led to the discovery that CD44 receptor and MMP-9 colocalize on cell membranes (31). Therefore, adhesion molecules can induce soluble MMPs to bind to cell

membranes and use their proteolytic activity to enhance cell invasion. Our study showed that CD44 crosslinking induced MMP-9 expression on breast tumor cell membranes and that CD44 receptors are used as a docking site to allow MMP-9 to exert protease activity on cell membranes thereby stimulating metastasis.

During CD44 crosslinking, protein A and mouse anti-CD44 mAb were used to form immunocomplexes. The expression of MMP-9 was detected on cell membranes after CD44 crosslinking. As described above, immunofluorescence revealed signs of MMP-9 and CD44 receptor association on the cell surface. In cytosol, this phenomenon was undetectable, which proves that MMP-9 induced by CD44 crosslinking did not form complexes with CD44 receptors in cytosol but interacted only at the cell surface. In the medium of the stimulated cells, low MMP-9 expression might be the soluble complexes of CD44 and MMP-9 formed through immunoprecipitation.

To analyze CD44 crosslinking-induced MMP-9 expression and its relationship to the invasive ability of human breast tumor cells, G8 myoblast was co-cultured with stimulated breast tumor cells or those treated with protease inhibitors. The invasive ability of human breast tumor cells increased after CD44 crosslinking, in that colonies grew larger and their outer edges became more ragged. This phenomenon was inhibited by the addition of the MMP inhibitors (GM6001 and 1,10-phenanthroline), which shows that MMP-9 is an active participant in the tumor invasion process. Because the above-mentioned inhibitors affect all MMPs, other cells were preincubated with rabbit anti-MMP-9 pAb before stimulating their CD44 receptors; the enhancement of tumor cell invasion ability of these cells was significantly inhibited. Clearly, the enhancement of invasive ability is directly related to MMP-9 expression. The co-capping of MMP-9 and CD44 receptors can be found on cell membranes where they promote the invasion and migration.

MMP-2, MMP-9, MMP-1, and MTI-MMP are known to be active in the progression of cancer, and MMP-2 and MMP-9 are often used as markers for breast and intestinal tumors (47,48). CD44 expression was shown to be elevated in migratory breast tumors as compared to normal breast tissue (49,50). We found that the invasive and migratory abilities of human breast tumor cells can be enhanced by CD44 crosslinking, which causes CD44 signaling, enhanced MMP-9 production on cell membranes and in the medium, and induction of co-capping with MMP-9. This study does not only contribute to our understanding of important molecular mechanisms of human breast tumor development and metastasis, but also provides novel thoughts that will fuel investigations dedicated to the development of therapeutic strategies to ameliorate breast cancer, such as the design of small molecules that interfere with the cellular relocation of MMP-9 upon CD44 receptor activation.

#### Acknowledgments

This work was supported in part by grant NSC 92-2320-B-010-036 from the National Science Council and a grant from the Ministry of Education, Aim for the Top University Plan, Taiwan, to H.-S. Wang.

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