

# Hyaluronan-CD44s Signaling Regulates Matrix Metalloproteinase-2 Secretion in a Human Lung Carcinoma Cell Line QG90<sup>1</sup>

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## Abstract

We investigated the production of matrix metalloproteinase (MMP) by hyaluronan (HA) stimulation in a human cancer cell line, QG90, that expresses a large amount of CD44s, a HA receptor. Treatment of QG90 with HA strongly activated MMP-2 secretion in a time- and dose-dependent manner. We found that expression of antisense CD44s in QG90 cells substantially inhibited the HA-dependent secretion of MMP-2, whereas overexpression of full-length CD44s augmented the HA-dependent secretion of MMP-2. In addition, pretreatment of cells with the neutralizing anti-CD44 antibody significantly inhibited both the HA-dependent MMP-2 secretion and the HA-dependent activation of mitogen-activated protein kinase in a dose-dependent manner. Similarly, treatment of cells with a Ras farnesyltransferase inhibitor, manumycin A, strongly inhibited the HA-dependent MMP-2 secretion. Moreover, *in vitro* invasiveness of QG90 and its activation by HA were clearly suppressed by the expression of antisense CD44s. In addition, treatment of cells with anti-CD44, a mitogen-activated protein/extracellular signal-regulated kinase kinase 1 inhibitor, PD98059, or phosphatidylinositol 3'-kinase inhibitors, wortmannin and LY294002, effectively blocked the HA-dependent activation of the invasiveness. In contrast, overexpression of full-length CD44 substantially activated the invasiveness of QG90. Taken together, HA-CD44s signaling plays a key role in the HA-dependent secretion of MMP-2 and, hence, in the invasiveness of QG90 cells.

## Introduction

MMPs<sup>3</sup> are a family of zinc-required matrix-degrading enzymes that play a critical role in invasion and metastasis of cancer cells (1). Despite its importance, the regulation of MMP secretion in cells is not yet completely understood. We have reported that stimulation of ovarian cancer cells with fibronectin strongly activated the secretion of MMP-9 via integrin-Ras signaling (2–4). These results suggest that activation of intracellular signaling cascade by an extracellular matrix component can activate MMP secretion.

To obtain more clues, we investigated the effect of HA on MMP production in cancer cells that express a large amount of CD44s, an HA receptor (5). The presence of high levels of CD44s is implicated in the unfavorable prognosis of human carcinomas (6, 7). Similarly, HA is emerging as an important metastatic maker in a number of human carcinomas (8–11). Inhibition of CD44 expression by an antisense oligonucleotide inhibited the invasion of glioma cells (11).

Overexpression of HA synthase 2, Has2, in a fibrosarcoma cell line activated anchorage-independent growth (12), whereas expression of HA synthase 1, Has1, in a mouse mammary carcinoma cell line activated metastasis (13). Despite the importance of HA-CD44 signaling in tumorigenesis, it has been studied mainly on the context of cell proliferation and migration (5, 14), and its role in the destruction of matrix remains to be studied. Here, we show that HA-CD44 signaling can activate MMP-2 secretion in a cell line derived from human small-cell lung carcinoma. Moreover, we show that both expression of antisense CD44 and treatment of cells with anti-CD44 can block the HA-dependent MMP-2 secretion and, subsequently, invasion of the cells.

## Materials and Methods

**Cell Culture, Plasmid Construction, and Transfection.** QG90 was cultured as described previously (15). Transfection of the plasmids into QG90 was performed as described previously (3). Drug-resistant colonies were selected as described previously (4).

**Isolation of CD44s Gene by Reverse Transcription-PCR and Preparation of Antisense CD44.** cDNA from QG90 was prepared by the TRIzol LS Reagent and Superscript Preamplification System (Life Technologies, Inc.). PCR was performed as described previously (16) using a specific pair of primers (5'-AGGGATCCTCCAGCTCCTTT-3'; 5'-TTTCCAAGATAATGTGTAG-3'). The PCR product was subcloned into pcDNA3.1/Myc-His B(-). For the preparation of antisense CD44 by PCR, a specific pair of primers (5'-AACAGTCGAAGAAGGTGTGGG-3'; 5'-TTTCCAAGATAATGGTGTAGGTGTTA-3') was used. The PCR products were resolved in a 1.5% agarose gel and subcloned into pcDNA3.1/Myc-His B(-).

**HA Treatment of Cells.** Cells were treated with HA (100 µg/ml; Sigma) in serum-free medium for the indicated times. To exclude the possibility of growth factor contamination, HA solution was boiled at 100°C for 5 min before use (14). After incubation, conditioned medium were collected, clarified by centrifugation, and subjected for zymography.

**Assay of Gelatin-degrading MMPs by Zymography.** Activities of MMPs in the conditioned medium were assayed by gelatin zymography as described previously (17).

**Immunoblotting.** Immunoblotting with anti-MMP-2 antibody was performed as described previously (18, 19).

**Invasion Assay by Matrigel.** Cells were assayed for their invasiveness by a modified Boyden chamber method (19). Briefly, conditioned medium obtained from QG90 were placed in the lower compartment of the chambers. Cells suspended in serum-free DMEM were seeded onto Matrigel-coated filters. After 15 h of incubation, cells invaded to the lower surface of the filters were fixed, stained, and counted.

## Results and Discussion

We first examined the expression of standard form CD44 protein (CD44s) in human cell lines derived from colon carcinoma (HT29), small-cell lung carcinoma (QG90), and gastric carcinoma (MKN28 and NUGC3) by immunoblotting with anti-CD44s (NeoMarkers, Fremont, CA). Among these cell lines, only QG90 expressed a large amount of CD44s, whereas others did not (Fig. 1A). We confirmed by cloning and sequencing that CD44 gene expressed in QG90 was

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<sup>3</sup> The abbreviations used are: MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; HA, hyaluronan; CD44s, standard form CD44; PI3K, phosphatidylinositol 3'-kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.

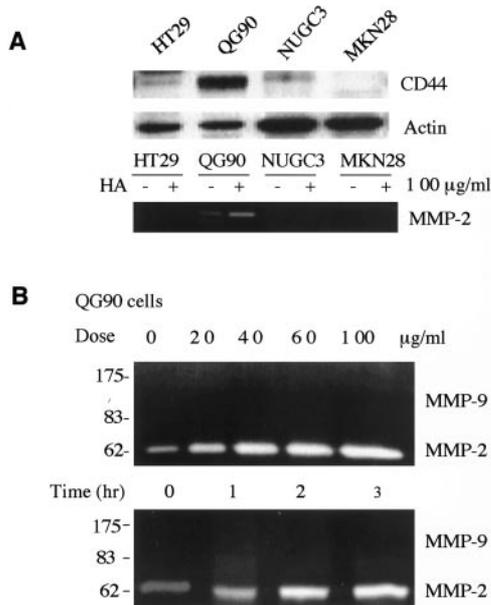


Fig. 1. Expression of CD44 and HA-dependent MMP-2 secretion in various human cancer cell lines. *A*, cell lysates from HT29, QG90, NUGC3, and MKN28 were probed with anti-CD44s or anti-actin (*top* and *middle* panels). Cells were incubated in serum-free medium with or without HA (100  $\mu$ g/ml) for 16 h. MMP activity in the conditioned medium was assayed by zymography. *B*, QG90 was incubated with serum-free medium containing indicated doses of HA for 3 h and MMP activity in the conditioned medium was assayed by zymography (*top* panel). Cells were incubated with serum-free medium containing HA (100  $\mu$ g/ml) for the indicated hours. Conditioned medium were collected and subjected to gelatin zymography (*bottom* panel).

exactly the standard form (data not shown). With these cells, we next examined MMP-2 secretion and effect of HA treatment on MMP secretion. Cells serum-starved for 15 h were incubated in serum-free medium for 3 h in the presence or absence of HA. After incubation, conditioned medium were collected and subjected to gelatin zymography. As shown in Fig. 1A (*lower* panel), QG90 secreted MMP-2 in the absence of HA and HA treatment activated the secretion whereas other cell lines did not secrete detectable amounts of MMP-2 nor respond to the HA treatment. In QG90, we found that HA stimulated MMP-2 secretion in a time- and dose-dependent manner (Fig. 1B). In contrast, gelatin-degrading activity corresponding to the size of MMP-9 ( $M_r$  92,000) was undetectable.

To study the role of CD44 in HA-dependent secretion of MMP-2, we established cells that overexpressed either antisense CD44s or full-length CD44s (Fig. 2). Antisense CD44s and full-length CD44s genes were transfected into QG90. Two independent clones in which CD44s expression was blocked by antisense (AS1 and AS2) and two clones that overexpressed full-length CD44s (WT1 and WT2) were selected and used for the study (Fig. 2A). Response of these clones to the HA treatment was examined (Fig. 2B). Cells were incubated for 15 h with or without HA, and conditioned medium were collected and subjected to zymography. In AS1 and AS2, basal levels of MMP-2 secretion were significantly suppressed, and HA treatment did not strongly activate the secretion. In contrast, MMP-2 secretion and its proteolytic activation were strongly activated in WT1 and WT2. Moreover, treatment of WT1 and WT2 with HA clearly augmented the secretion of MMP-2. Intracellular levels of MMP-2 of these cell lines in the presence or absence of HA were examined by immunoblotting of cell lysates with anti-MMP-2. We found that MMP-2 production in AS1 and AS2 was substantially suppressed (Fig. 2B, *lower* panel).

To confirm these observations, we examined the effect of neutralizing antibody against CD44 in HA-dependent MMP-2 secretion. We

found that pretreatment of QG90 with anti-CD44, J173 (Immunotech), clearly inhibited the activation of MMP-2 secretion by HA treatment in a dose-dependent manner (Fig. 3A, *upper* panel). These results strongly suggest that CD44 is required for the activation of MMP-2 secretion by HA in QG90 cells. Accumulated evidence suggests that HA-CD44 binding activates the Ras-MEK1-MAPK signaling pathway (5, 14). Indeed, we found that HA stimulation activated the phosphorylation of MAPK in QG90 cells. In addition, pretreatment of cells with neutralizing antibody significantly blocked the activation of MAPK by HA (Fig. 3A, *middle* panel). To confirm these observations, we examined the effect of manumycin A, a potent inhibitor of Ras farnesyltransferase (19). As shown in Fig. 3B (*lower* panel), activation of MMP-2 secretion by HA was substantially blocked by treatment with manumycin A, suggesting the requirement of Ras-MEK1-MAPK signaling in HA-dependent activation of MMP-2 secretion.

We next investigated the effect of HA stimulation on the invasive-

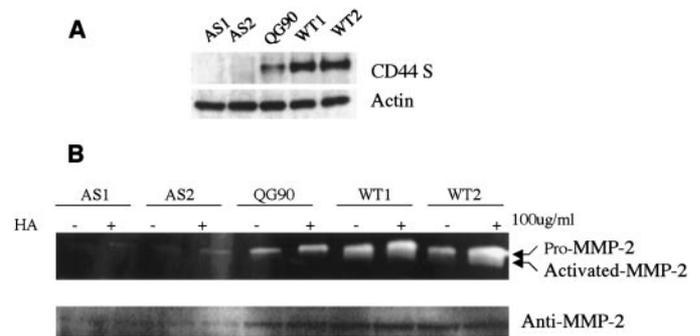


Fig. 2. Inhibition of HA-dependent MMP-2 secretion by antisense CD44. *A*, expression of CD44s and actin in cells transfected with antisense CD44 gene (AS1 and AS2) or full-length CD44s gene (WT1 and WT2). Transfected cells were examined by immunoblotting with anti-CD44 (*top* panel) or antiactin (*bottom* panel) to compare with those of parental QG90. *B*, cells were cultured in the presence (+) or absence (-) of HA for 15 h, and MMP-2 activity in the conditioned medium was examined by zymography (*top* panel). After incubation, cells were harvested and relative amounts of intracellular MMP-2 were examined by immunoblotting with anti-MMP-2 (*bottom* panel).

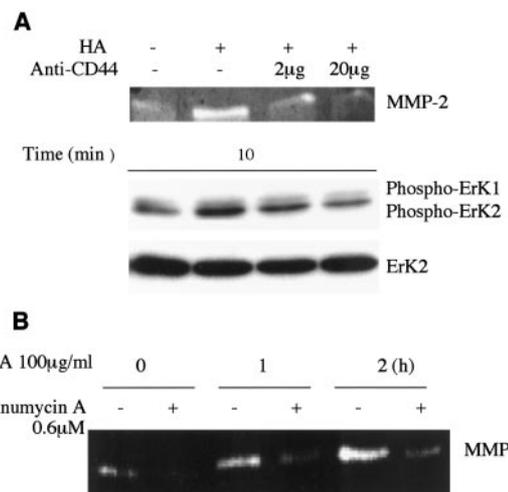


Fig. 3. Inhibition of HA-dependent MMP-2 secretion and MAPK activation by anti-CD44s antibody and manumycin A. *A*, QG90 cells were pretreated with anti-CD44 at the concentrations of 0, 2, or 20  $\mu$ g/ml for 3 h, and stimulated with HA for an additional 3 h in the presence or absence of anti-CD44. Conditioned medium were harvested and subjected to zymography (*top* panel). QG90 cells pretreated with indicated amounts of anti-CD44 for 3 h were stimulated with HA (100  $\mu$ g/ml) for 10 min and harvested. Cell lysates were subjected to immunoblotting with anti-phospho-MAPK (*middle* panel) or anti-ERK2 (*bottom* panel). *B*, QG90 cells treated (+) or untreated (-) with 0.6  $\mu$ M manumycin A for 3 h were incubated with 100  $\mu$ g/ml of HA for the indicated hours. MMP-2 activity in the conditioned medium was assayed by zymography.

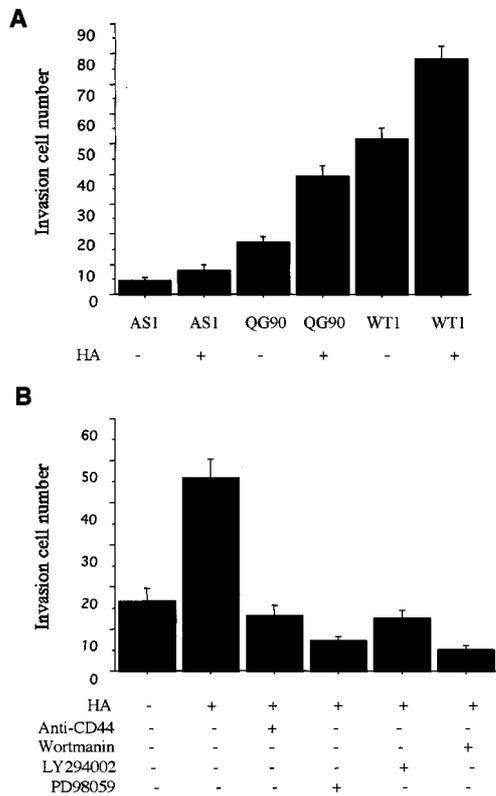


Fig. 4. Suppression of *in vitro* invasiveness of QG90 cells by antisense CD44 and pretreatment with anti-CD44 or inhibitors. A, the invasive ability of parental QG90 and QG90 expressing either antisense CD44 (AS1) or full-length CD44s (WT1) was assayed in the presence (+) and absence (-) of HA (100  $\mu$ g/ml) by a modified Boyden chamber method (*top graph*). B, QG90 cells were pretreated with anti-CD44 (10  $\mu$ g/ml), wortmannin (100  $\mu$ M), LY294002 (10  $\mu$ M), or PD98059 (50  $\mu$ M) for 3 h. After pretreatment, HA-dependent invasiveness of the cells in the presence of antibody or drugs was assayed by a modified Boyden chamber method; bars,  $\pm$ SD.

ness of QG90 and the effect of antisense CD44s expression on HA-dependent invasiveness by a method with the reconstituted basement membrane, the Matrigel invasion chamber method (17). QG90, QG90-expressing antisense CD44s (AS1) and QG90 overexpressing full-length CD44s (WT1) were treated or untreated with HA and loaded onto the upper chambers. After an 8-h incubation, cells that penetrated the membranes were fixed, stained, and counted. As shown in Fig. 4 (*upper graph*), the invasiveness of QG90 was clearly activated by HA treatment. In contrast, the invasiveness of AS1 cells was significantly suppressed and poorly responded to the HA stimulation. On the other hand, the invasiveness of WT1 was substantially higher than that of QG90 and clearly activated by HA-stimulation.

We next assayed the effect of anti-CD44 neutralizing antibody, J173, on the invasiveness of QG90 (Fig. 4, *lower graph*). Compared with control HA-stimulated QG90, pretreatment of cells with the antibody substantially inhibited the HA-dependent invasiveness of QG90. These results suggest that HA-dependent activation of the invasiveness of QG90 requires HA binding to CD44.

Accumulated evidence suggests that HA-CD44 binding activates the Ras-MEK1 and the PI3 kinase-Akt signaling pathways (5, 14). In addition, we found that Ras-MAPK signaling played a critical role in the activation of MMP-2 secretion by Con A (18) and by v-Crk (19). Indeed, we observed the HA-dependent activation of MAPK and its inhibition by neutralizing anti-CD44 antibody as well as inhibition of HA-dependent MMP-2 secretion by manumycin A in QG90 (Fig. 3). To obtain more clues, we investigated the effects of various inhibitors on the invasiveness of QG90 cells (Fig. 4, *lower graph*). PD98059 is a potent inhibitor of MEK1. We found that treatment of QG90 with

PD98059 strongly suppressed the HA-dependent activation of the invasiveness, suggesting the important role of MEK1 signaling in the activation of invasiveness. In addition to MEK1, we next examined the involvement of another signaling pathway, PI3k, on HA-dependent activation of the invasiveness, because we have found the activation of PI3k by HA that stimulated the motility of v-src-transformed cells (14). Wortmannin and LY294002 are potent inhibitors of PI3k (19). We found that pretreatment of QG90 cells with either wortmannin or LY294002 efficiently suppressed the HA-dependent activation of the invasiveness. These results suggest that HA-CD44 signaling requires the dual pathways, MEK1-MAPK and PI3k, to activate the invasiveness of QG90.

In this paper, we showed that HA-CD44s signaling regulates MMP-2 secretion and invasiveness of QG90 cells in a Ras signaling-dependent manner. The activation of MMP-2 secretion and invasiveness by HA could be blocked by the expression of antisense against CD44s. Moreover, we found that pretreatment of cells with the neutralizing antibody against CD44 strongly blocked both the invasion of cells. These results suggest the critical role of CD44s in HA-dependent activation of MMP-2 secretion and invasiveness of QG90 cells. In addition, we showed that activation of the invasiveness by HA required the dual signaling pathways, MEK1-MAPK and PI3k. Inhibition of either one of these pathways by specific inhibitors strongly suppressed the HA-dependent invasion of the cells. Thus, our results may provide a new therapeutic target for tumor invasion through the regulation of the CD44 signal pathway.

In contrast to HA stimulation of QG90 cells, we have reported previously that fibronectin stimulation of ovarian cancer cells activated the secretion of MMP-9 rather than MMP-2 in a Ras-dependent manner (2-4). Both HA-dependent MMP-2 secretion in QG90 and fibronectin-dependent MMP-9 secretion in ovarian cancer cells require the MAPK and the PI3k signaling pathways. Whether another signaling pathway specific for each receptor determines the specificities of MMP to be secreted remains unclear. Additional studies including the identification of signaling molecules between CD44 and MEK1/PI3k are required for the complete comprehension of HA-CD44-dependent tumor invasion.

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