ANTICANCER RESEARCH *24*: 489-494 (2004)

Increased Invasion and Expression of MMP-9 in Human Colorectal Cell Lines by a CD44-dependent Mechanism

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Abstract. *Background: In order to investigate the interactions between MMPs and CD44 we stably transfected a non-invasive colon cell line, SW480 with the cDNA for MMP-9 and investigated the effect on CD44 expression and in vitro invasion and migration. Materials and Methods: In vitro invasion and migration assays were carried out using Biocoat matrigel invasion chambers. MMP and CD44 expression was determined using zymography, Western blot analysis and RT-PCR. Results: Transfection of the parental SW480 cells with the cDNA for MMP-9 (SW480M9) caused increased invasion and migration. MMP-9 expression increased when the SW480M9 cells were grown on HA and collagen and cultured in the presence of a CD44-activating antibody. Treatment of the cells with HA and a CD44-activating antibody also resulted in increased invasion, migration and attachment. Conclusion: These results demonstrated that CD44 and MMP interactions are important in controlling tumour cell invasion and migration.*

The matrix metalloproteinases (MMPs) are a family of highly conserved zinc-dependent endopeptidases which collectively are capable of degrading components of the extracellular matrix (ECM). The MMP family currently contains 23 members that can be divided into at least 5 classes based on substrate specificity: gelatinases, collagenases, stromelysins, membrane-type MMPs and others (1). They have been shown through numerous studies to be key role players in both normal and disease processes, but it is their role in tumour invasion and metastasis which has been most extensively studied (2). Metastasis is the spread of tumour cells from a primary tumour to distant sites in the body and is the most lethal aspect of cancer.

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Key Words: MMP-9, CD44, invasion, migration, colon tumour cell lines.

MMP activity has been implicated at almost every stage of the metastatic cascade from local invasion at the primary tumour site to growth and development of a tumour at a secondary site (2). The substrate specificities of the two gelatinases, MMP-2 and MMP-9, differ from other MMPs in that they can degrade type IV collagen which is present in the basement membrane (1). They have been shown in several studies to function as independent predictors of disease outcome and recurrence (3).

CD44, the major cell surface receptor for hyaluronic acid (HA), is a multifunctional transmembrane adhesion molecule that also plays a key role in cell signalling (4). CD44 mediates cell-cell and cell-matrix interactions and is involved in adhesion, migration and invasion. It has been estimated that 45 CD44 variants exist as a result of alternative mRNA splicing (5). Abnormal CD44 variant expression has been associated with tumour progression and over-expression of CD44v6 has been observed more often in advanced colon cancers (Dukes' stage C/D) compared with Dukes' A/B, and it can therefore be used as a prognostic marker (6).

Both MMPs and CD44 are critical to the invasive process and several studies have recently begun to define how these molecules interact with each other. Stimulation of CD44 has been shown to increase MMP-2 expression in human melanoma cells (7) and in lung carcinoma cells (8). MMP-9 has been shown to associate with CD44 on the surface of mouse mammary carcinoma and human melanoma cells (9). CD44v3 can anchor MMP-7 on the cell surface and target its activity (10). A recent paper has also shown that MT1-MMP can cleave CD44 and promote cell migration in a pancreatic tumour cell line (11).

Appreciating the importance of both attachment to and degradation of the ECM in tumour cell invasion, it is no longer appropriate to consider CD44 and MMPs as acting in isolation. In this study the interaction between CD44 and MMP-9 in regulating invasion and migration in colorectal tumour cell lines was examined. The non-metastatic SW480 cell line was stably transfected with the cDNA for MMP-9. The effect of MMP-9 on cell adhesion and migration was

assessed and cells were grown on various ECM components to study the effect on MMP activity. Activation of CD44 through ligand binding and antibody treatment caused increased MMP expression and invasion. Inhibition of CD44 activity by antibody treatment resulted in decreased invasion. These results demonstrated the importance of MMP and CD44 interactions in regulating colon tumour cell invasion.

Materials and Methods

Cell culture and transfection of SW480 cells. The cell lines used in this study were SW480, (ATCC# CCL-228) established from a primary human colon adenocarcinoma, SW620 (ATCC# CCL-227), established from a lymph node metastasis from the same patient as the SW480 and BHK92 (BHK cells transfected with the cDNA for MMP-9, a gift from Dylan Edwards, University of East Anglia). All cell lines were maintained in Dulbecco's modification of Eagles' medium (DMEM) supplemented with 5% foetal calf serum (FCS) in a humid 5% CO_2 atmosphere at 37˚C. The SW480 cells were transfected with 10μg pCMV92 (human MMP-9 cDNA sub-cloned into pCMV expression vector) as previously described (12) and 2 μg pSV2neo using Lipofectamine (Invitrogen Paisley, UK) following the manufacturer's instructions. Cells were selected in 600 μg/ml Geneticin (G-418) for 4 weeks. Individual colonies were selected using cloning cylinders (Sigma, Dublin, Ireland).

Zymography. Conditioned media (CM) was collected by incubating 80% confluent cultures for 72 h in serum-free media. CM was concentrated using Microcon™ centrifugal filter devices (Amicon, Cork, Ireland) and protein concentration determined using the bicinchoninic acid (BCA) assay (Pierce, Tattenhall, UK). Gelatinase activity was assessed in 30 μg total protein as previously described (13). To investigate the effect of CD44 stimulation on MMP activity, cells were incubated with 50 μg/ml CD44 activating antibody $F_{10\text{-}44\text{-}2}$ (Biodesign, Saco, ME, USA) or with mouse IgG (Sigma) as control in serum-free media. Cells were also treated with 0.25mg/ml HA, 0.01mg/ml Collagen type IV or 0.3 μg/ml anti-β1-integrin stimulating antibody (Sigma) in serum-free media for 72 h.

Western blot analysis. Western blot analysis of concentrated CM (as prepared for zymography) was carried out for detection of MMP-2 and MMP-9. Western blot analysis for detection of CD44 was carried out on total cell lysates prepared as previously described (14). Equal amounts of protein (50μg) were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred and blocked in 5% dried milk in TBST (0.15M NaCl, 0.01M Tris, 0.05% (v/v) Tween-20). Primary antibodies were diluted; D2.1 mouse pan specific anti-CD44 (a gift from Aideen Long, Royal College of Surgeons in Ireland) 1/1000, MAC96 mouse anti-MMP-9 (a gift from Andrew Docherty, Celltech) 1/500, GL8 mouse anti-MMP-2 (a gift from Andrew Docherty, Celltech, Dublin, Ireland) 1/2000 and mouse anti-actin (Merck Biosciences, Nottingham, UK) 1/10,000 in the same blocking solution and incubated overnight at 4˚C. The blots were washed, incubated with anti-mouse-horseradish peroxidase (HRP) (Amersham, Little Chalfont, UK) 1/5000 in TBST for 2 h at room temperature and then developed using the enhanced chemoluminesence (ECL) system (Pierce).

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from equal cell numbers using TRI REAGENT (Sigma) following the manufacturer's instructions. Reverse transcription was as previously described and 5μl of the resultant cDNA was used as template for PCR (13). The sequences for primers with annealing temperatures indicated in brackets were as follows: MMP-9-5' ATG AGT TCG GCC ACG CGC TGG GCT T 3': 3' T GCC GGT GAT GAC ACG GAA ACT CAG 5' (54 ˚C); β-Actin-5' TCA GGA GGA GCA ATG ATC TTG A 3': 3' TC GAA GAG GAA TTA CAG TGC GTG CTA AAG 5' (55˚C). The number of PCR cycles used was 30 and the expected product size after primer amplification was as follows: MMP-9: 329 base pairs (bp) and β-actin: 682bp.

In vitro invasion and migration assays. Biocoat™ matrigel invasion chambers (Becton Dickinson Labware, UK) were used to test the *in vitro* invasive activity of the cell lines. $5x10^5$ cells were seeded into the upper chamber and incubated for 24 h at 37˚C. Membranes were fixed and stained as previously described (13) and invasive cells counted in five 10X magnification fields. Results were expressed as average cell count per field. To investigate the effect of HA on cell invasion, cells were seeded in serum-free media containing 0.25mg/ml HA in both chambers. SW480M9 cells were allowed to invade in the presence of 50 μg/ml, CD44-activating antibody $F_{10\text{-}44\text{-}2}$ (Biodesign), CD44-blocking antibody IM7.8.1 (gift from Aideen Long, Royal College of Surgeons, in Ireland), or mouse IgG (Sigma) as a control. Migration assays were carried out under the same conditions except in the absence of matrigel. All experiments were done in duplicate and were repeated three times.

Adhesion assays. To investigate the adhesion of cells to various ECM components, a flat-bottomed 96-well plate was coated overnight at 4˚C with either 1mg/ml HA or 50 μg/ml fibronectin (FN). The plate was washed twice with phosphate-buffered saline (PBS), blocked with 1mg/ml bovine serum albumin (BSA) for 2 h at 4˚C and then $1x10^5$ cells were added and allowed to adhere for 90 min at 4˚C. Residual media was aspirated and the adherent cells were incubated in media containing [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS reagent) (Promega, Southampton, UK). Absorbance was read at 490nm after incubation at 37˚C for 3 h. The absorbance value obtained by seeding untreated wells (*i.e.* media was not aspirated) represented 100% adhesion and all other values were divided by this to calculate percentage adhesion. Experiments were performed in triplicate on three separate occasions and T-tests performed to ensure significance.

Results

Over-expression of MMP-9 in the SW480 cells and characterisation of colon cell lines. Following transfection of the SW480 cells with the cDNA for MMP-9, a total of 20 clones were selected and characterized. Clone 11 (referred to as SW480M9) was chosen for further analysis as it secreted the highest levels of MMP-9 protein as shown by gelatin zymography (Figure 1A). SW480M9 showed a marked increase in MMP-9 levels in comparison to the parental SW480 and its lymph node metastatic derivative SW620. The identity of MMP-9 was confirmed by Western

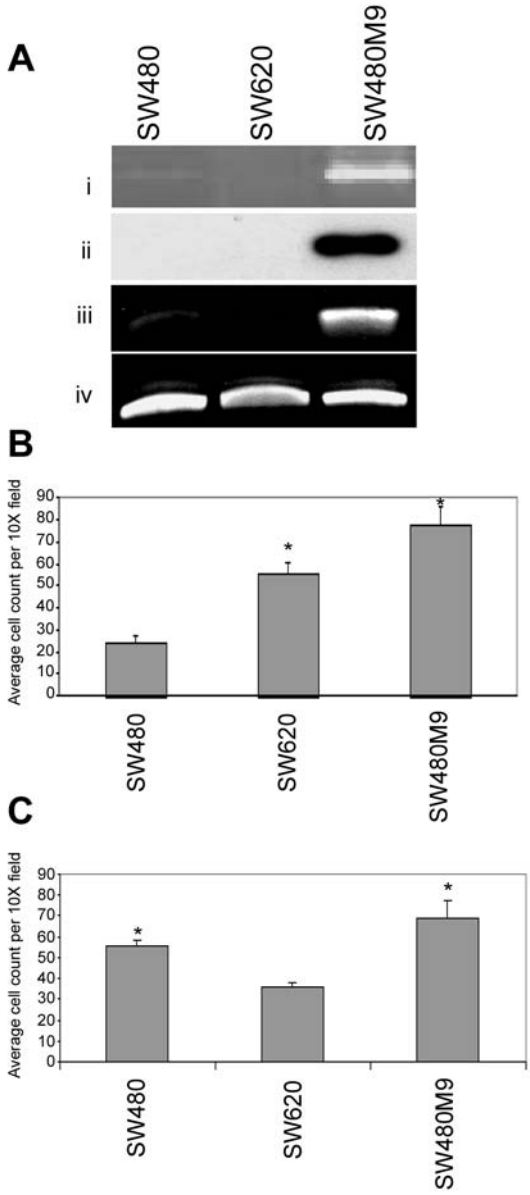

Figure 1. *Characterisation of colon cell lines. (A) MMP-9 expression in SW480, SW620 and SW480M9 cells was analysed by; (i) gelatin zymography, (ii) Western blot, (iii) RT-PCR with (iv) RT-PCR for β actin as a control. (B) Comparison of in vitro invasion of the SW480, SW620 and SW480M9 cells. (C) Comparison of in vitro migration of cell lines. The asterisk denotes that p<0.005.*

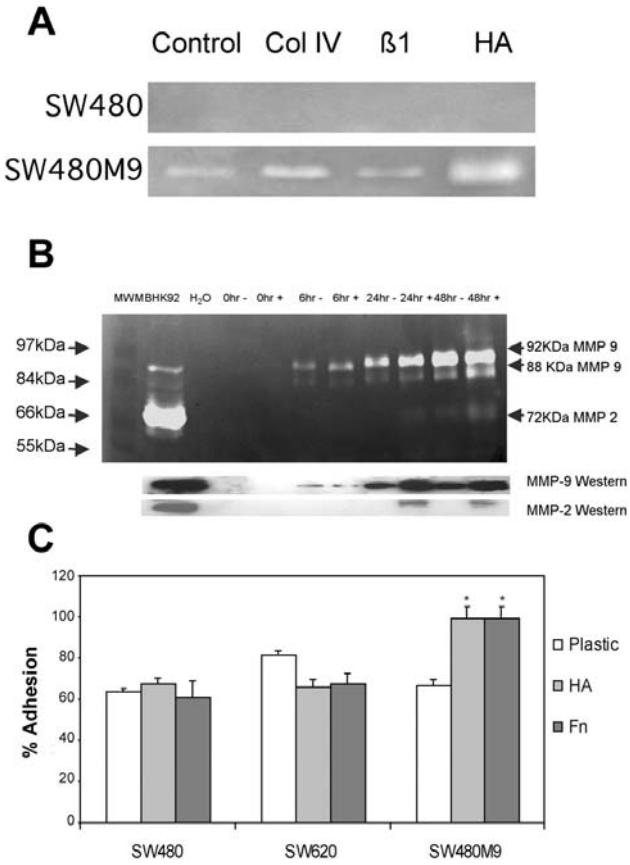

Figure 2. *The effect of CD44 stimulation on MMP-9 expression. (A) MMP-9 expression in SW480 and SW480M9 cells grown in the presence of type IV collagen, HA and β_1-integrin-activating antibody. (B) MMP-9 and MMP-2 expression in the SW480M9 cells treated with CD44-activating antibody over 48 h by zymography and Western blot using BHK92 as a positive control. (C) Comparison of cell adhesion to plastic, HA and FN. The asterisk denotes that p<0.005.*

blot analysis (Figure 1a) using an MMP-9 specific antibody. MMP-9 was not detected in either the SW480 or SW620 cells by Western blot analysis. RT-PCR data (Figure 1A) confirmed over-expression of MMP-9 in the SW480M9 cells at the mRNA level when compared to the SW480 and the SW620, which only showed faint bands. β-actin was used as an equal loading control for RT-PCR.

Since ECM degradation is key to tumour cell invasion, the *in vitro* invasiveness of these cell lines through matrigel-coated membranes was compared. The SW480 cells showed little invasion (Figure 1B) in comparison to the SW620 and SW480M9 cells, which were significantly ($p<0.005$) more invasive. The SW620 and SW480M9 were respectively twice and three times more invasive. The migratory capacities of the cells were determined using the same procedure as the *in vitro* invasion assays in the absence of matrigel. Over-expression of MMP-9 in the SW480M9 cells increased their migratory capacity in comparison to the parental SW480 cells (Figure 1C). However, the parental SW480 cells were significantly ($p<0.005$) more migratory than the SW620, which were established from a lymph node metastasis from the same patient.

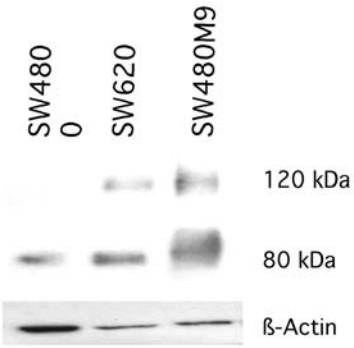

Figure 3. *CD44 expression in colon cell lines. CD44 expression profiles of the SW480, SW620 and SW480M9 cell lines by Western blot analysis using β-actin as an equal loading control.*

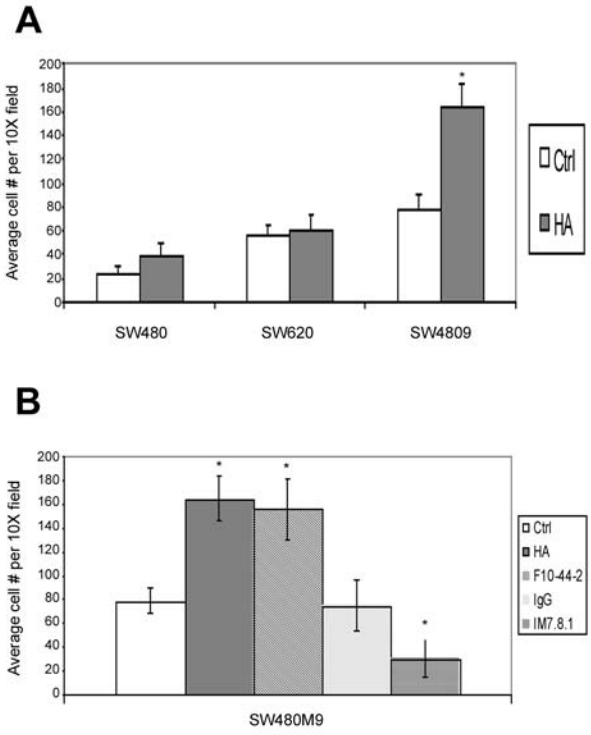

Figure 4. *The effect of enhanced expression of MMP-9 via CD44 on in vitro invasion. (A) In vitro invasion of the SW480, SW620 and SW480M9 cells in the presence of HA. (B) The in vitro invasion of the SW480M9 cells in the presence of HA, CD44-activating antibody ($F_{10\text{-}44\text{-}2}$), mouse IgG (as a control) and CD44-blocking antibody (IM7.8.1). The asterisk denotes that $p<0.005$.*

Effect of ECM components and CD44 activation on MMP-9 expression and cell adhesion. To investigate the effect of ECM components and integrin signalling on MMP-9 expression, cells were cultured in the presence of HA, type IV collagen and a β_1-integrin-activating antibody. After treatment, neither the SW480 or SW620 cells, which did not originally secrete measurable levels of MMP-9, showed any increase in MMP-9 expression levels (Figure 2A and data not shown). The SW480M9 cells showed no increase in MMP-9 activity in the presence of the β_1-integrin-stimulating antibody and a slight increase when grown on type IV collagen. Growth of the SW480M9 cells on HA greatly increased the levels of MMP-9. Since CD44 is the major cell surface receptor for HA, the SW480M9 were treated with an anti-CD44 monoclonal antibody ($F_{10\text{-}44\text{-}2}$). Instead of blocking its activity, $F_{10\text{-}44\text{-}2}$ activates CD44, mimicking its ligation of HA in the ECM and subsequently activating its associated intracellular signalling pathways. CM from antibody-treated cells was collected at timed intervals and analysed for MMP-9 expression. Within 6 h of treatment with the CD44-activating antibody, cells showed a slight increase in MMP-9 activity (Figure 2B) when compared with cells treated with a control mouse IgG. This difference in activity was more obvious after 24 and 48 h. Interestingly, activation of pro-MMP-9 to its active 88 kDa form occurred after 24 and 48 h. MMP-2 activity was also detected after 24 and 48 h of CD44 activation. These observations were confirmed by Western blot analysis (Figure 2B) with specific MMP-2 and MMP-9 antibodies.

To further investigate the behaviour of cells in the presence of ECM components, adhesion assays were carried out in the presence of HA and FN. Adhesion is a key event in the metastatic process where cells must first adhere to the ECM prior to its degradation. Increased cell-cell signalling and contact is also mediated by increased expression of cell adhesion molecules. The SW480M9 cells, which were the most responsive to HA treatment, also showed the most adherence to FN and HA (Figure 2C). All percentage adhesion values were significant ($p<0.005$).

Expression and up-regulation of CD44 in MMP-9 transfected cells. Due to the increased adhesion of the SW480M9 cells on HA and the enhancing effect of HA and CD44 stimulation on MMP-9 activity, the expression of CD44 protein was examined. All cell lines studied expressed the 80kDa CD44 (Figure 3), often referred to as CD44s or standard CD44. The SW480M9 cells expressed the highest levels of CD44s compared to the SW480 and SW620 cells. Interestingly, the metastatic SW620 and the more invasive SW480M9 showed expression of a 120kDa variant of CD44 with SW480M9 showing highest levels of expression. These differences in CD44 expression may help explain the increased adhesion of SW480M9 to HA as well as the effect HA had on MMP-9 activity in these cells.

Effect of CD44 activation on in vitro invasion. To show that CD44-mediated up-regulation of MMP-9 expression had an effect on cell invasion, the *in vitro* invasion of the cells in the presence of HA was assayed. The presence of HA increased invasion in all cell lines tested (Figure 4A). The most dramatic increase was seen with the SW480M9 cells where twice as many cells ($p<0.005$) invaded when treated with HA. Therefore, the effect of a CD44-activating antibody on invasion was studied. CD44 activation increased the *in vitro* invasion of SW480M9 cells two-fold ($p<0.005$) (Figure 4B) when compared with the invasion of the same cells treated with control mouse IgG. A CD44-blocking antibody (IM7.8.1) was used to further confirm that the increased invasion was CD44-dependent. Blocking of CD44 with this antibody resulted in a significant reduction ($p<0.005$) in invasion (Figure 4B).

Discussion

In this study the interactions between CD44 and MMP-9 in regulating invasion and migration in human colorectal cancer cell lines was investigated. The non-metastatic SW480 cell line, established from a primary human colon adenocarcinoma, was stably transfected with the cDNA for MMP-9. Over-expression of MMP-9 in a rat transformed embryo cell line has previously been shown to increase the invasion and migration of the cells (15). This paper was the first report of MMP-9 over-expression in a human colon cell line. The parental SW480 cells do not express MMP-9 and transfection of the cDNA for MMP-9 into these cells increased their invasion and migration. Interestingly, the SW620 cells, which were established from a lymph node metastasis from the same patient as the SW480, also did not express MMP-9. The MMP-9-transfected cells also showed increased migratory capacity. Surprisingly, the parental SW480 cells were more migratory than the SW620. A similar result was also obtained by Kubens and Zanker using a three-dimensional collagen matrix and time-lapse video recording assay (16).

Within the past few years, many researchers have demonstrated the importance of MMP interactions with cell adhesion molecules in regulating tumour cell invasion and metastasis. Several investigators have shown the importance of integrins in regulating MMP expression *e.g.* the αVβ6 integrin promotes invasion of squamous carcinoma cells through up-regulation of MMP-9 (17). Stimulation of CD44 has been shown to increase MMP-2 expression in human melanoma cells (7) and in lung carcinoma cells (8). In this study we showed that culturing colon cells on ECM components increased MMP-9 expression. HA, the major receptor for CD44, caused the greatest increase. In addition, direct stimulation of CD44 by treating with an activating antibody ($F_{10\text{-}44\text{-}2}$) caused an increase in MMP-2 and MMP-9 expression and also caused activation of MMP-9 to its active form. This is the first report to show up-regulation of MMP-9 expression following CD44 stimulation in colon cells.

We also looked at adhesion of the cells on various matrices including, plastic, FN and HA. The MMP-9-transfected cells attached better to FN and HA than any of the other cell lines. Examination of the levels of CD44 protein expressed in the cell lines showed that all three cell lines expressed the standard form of CD44. An additional higher molecular weight band was seen in the metastatic SW620 and the MMP-9-transfected cell line. This band had a molecular weight of ~120kDa and could be CD44v6, which has been previously been shown to be involved in colon metastasis (6). Exon-specific PCR would be needed to confirm the identity of this band.

Since we had shown that growth of the SW480M9 cells on HA and direct stimulation of CD44 caused an increase in MMP activity, we then looked at whether this increase in MMP-9 activity had a functional effect on cell invasion and migration. Stimulation of CD44 by treatment with HA and a CD44-activating antibody caused an increase in the invasion of the SW480M9 cells, which could be inhibited by treatment with a CD44-blocking antibody.

These results suggest that CD44 activation can directly increase the levels of MMP-9 and cause an increase in cell invasion in colon cell lines. Interestingly, Yu and Stamenkovic (9) have shown that MMP-9 can associate with CD44 on the surface of mouse mammary carcinoma and human melanoma cells. This association may cause activation of signal transduction molecules, *e.g.* HA-CD44 binding activates the ras-MEK1 and the PI3 kinase-Akt signalling pathways in a human lung carcinoma cell line (8). Future experiments will determine the signalling mechanisms involved in the up-regulation of MMP-9 expression in these cells. In addition, using MMP-9 promoter constructs, we will attempt to elucidate the transcriptional mechanisms involved in regulating MMP-9 gene expression in these cells.

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Received September 17, 2003
Accepted January 5, 2004