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SUPPRESSION OF MATRILYSIN INHIBITS COLON CANCER CELL INVASION *IN VITRO*

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Matrilysin is a member of the matrix metalloproteinase gene family, which is believed to play an important role in tumor invasion and metastasis. We examined the effects of over- and under-expression of matrilysin on the ability of colon cancer cells to migrate across an artificial membrane *in vitro*. Introduction of matrilysin caused colon cancer cells to become more invasive as assessed by an *in vitro* invasion assay. In contrast, expression of matrilysin was down-regulated by all *trans*-retinoic acid or by introduction of anti-sense matrilysin in BM314 colon cancer cells. This down-regulation caused these cells to become less invasive. We demonstrated a correlation between matrilysin level and the invasive potential of human colon cancer cells, implying an important role for matrilysin in the control of tumor invasion *in vitro*.

Evidence has emerged that proteolytic degradation of the extracellular matrix is required for tumor cells to invade basement membranes, stromal matrix and cell junctions. Cancer cells secrete various proteinases, including metalloproteinase, serine proteinases, thiol proteinases and aspartic proteinases. There is substantial evidence that the matrix metalloproteinases (MMPs) play a particularly important role in tumor progression (Liotta and Stetler-Stevenson, 1990; Goldberg and Eisen, 1991). In the past, the involvement of MMPs in tumor progression has been demonstrated using their inhibitors, tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), TIMP-2 and estramustine (Declerck *et al.,* 1992; Tsuchiya *et al.,* 1993). Direct evidence linking expression of MMP to the metastatic phenotype in transformed rat embryo cells has been reported (Bernhard *et al.,* 1994).

Matrilysin (MMP-7) is a member of the MMP gene family and exhibits a wide spectrum of substrate specificity (Quantin *et al.,* 1989; Miyazaki *et al.,* 1990). Matrilysin has been shown to activate gelatinase A (MMP-2) *in vitro* (Quantin *et al.,* 1989). This smallest metalloproteinase has only 2 structural domains and lacks a C-terminal domain, which contributes to its capacity to bind the natural inhibitor TIMP. Therefore, matrilysin is believed to resist inhibition by TIMP *in vivo* (Baragi *et al.,* 1994). Matrilysin also plays an important role in inactivation of serpins (serine proteinase inhibitors) in malignant tissues, where a cascade of metalloproteinases and serine proteinases is required for tissue degradation (Zhang *et al.,* 1994). Previous studies have shown that matrilysin mRNA is expressed in various cancers, including squamous-cell carcinoma of the head and neck or of the lung, and in breast, prostate, stomach and colorectal carcinomas (Yoshimoto *et al.,* 1993; McDonnell *et al.,* 1994). Over-expression of matrilysin in the human prostate tumor-cell line DU-145 increased invasive potential in the SCID murine model (Powell *et al.,* 1993).

In colorectal cancers matrilysin is produced by cancer cells themselves, while other MMPs, including MMP-1, MMP-2, MMP-9 and MMP-11, are produced by stromal cells (Pyke *et al.,* 1993). This suggests that matrilysin may directly contribute to invasion of colon cancer cells. Therefore, we modulated the expression of matrilysin in colon cancer cells by introducing both sense and anti-sense matrilysin and tested the effects of this modulation on the ability of colon cancer cells to migrate across an artificial membrane *in vitro.* We also found the down-regulator of matrilysin expression and tested its effect on the invasiveness of colon cancer cells. Modulation of matrilysin expression markedly affected *in vitro* invasive potential, suggesting that matrilysin plays an important role in invasion of colon cancer cells.

MATERIAL AND METHODS

Cell cultures and treatments

All colon cancer cell lines used for these studies were obtained from the Japanese Cancer Research Resources Bank. CHC-Y1 and DLD-1 were maintained in RPMI 1640 containing 10% FBS. BM314 cells were maintained in Dulbecco's modified minimum essential medium containing 10% FBS.

BM314 cells (8×10^5) were seeded per 100-mm dish. For serum starvation, cells were placed into serum-free medium for 12 hr before adding TGF-β (0.1 ng/ml; R&D Systems, Minneapolis, MN), or of all *trans*-retinoic acid (ATRA) (1 μM; Sigma, St. Louis, MO). Total RNA was isolated at 24, 48 and 72 hr after addition of these compounds.

DNA transfection

A full-length cDNA encoding human matrilysin was subcloned into the eukaryotic expression vector pcDNAI neo (Invitrogen, San Diego, CA), which contains the cytomegalovirus promoter/enhancer for expression and a neomycin resistance gene allowing G418 selection. Both sense (pcDNAI Mat) and anti-sense (pcDNAI Mat-as) orientations were prepared. Each was introduced into CHC-Y1 or DLD-1 cells by co-precipitation with calcium phosphate, and stable G418-resistant clones were isolated and expanded. Transfectants containing pcDNAI Mat-as were used as controls and designated CHC-co or DLD-co.

PcDNAI Mat-as was introduced into BM314 cells, which constitutively expressed matrilysin mRNA. The extent of stable anti-sense RNA expression and its effect on the endogenous matrilysin mRNA level was investigated by Northern blot analysis using strand-specific probes to identify sense or anti-sense matrilysin RNA (Yamamoto *et al.,* 1994). Transfectants containing the selection plasmid pcDNAI neo alone were used as controls and designated BM-co.

Northern blot analysis

Total RNA from cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform method. Northern analyses were performed on 10 μg of total RNA. The membrane was hybridized with a matrilysin cDNA probe labeled by the random primer method in 50% formamide/5× Denhardt's solution/3× SSC/100 μg/ml salmon sperm DNA/1% SDS at 42°C overnight. It was then washed twice in 2× SSC/0.1% SDS at room temperature for 10 min and 3 times in 0.1× SSC/0.1% SDS at 55°C for 15 min. The membrane was also stripped and reprobed with β-actin cDNA for RNA loading control.

Casein zymography

Zymography in SDS-polyacrylamide gel containing casein was performed as previously described (Miyazaki *et al.,* 1990).

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The cell number–adjusted aliquots of the culture media from transfectants grown for 24 hr in serum-free medium were electrophoresed in a 10% polyacrylamide gel embedded with 1 mg/ml casein. After electrophoresis, gels were washed in 2.5% Triton-X 100 for 1 hr to remove SDS. Gels were then incubated for 18 hr at 37°C in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and 0.02% NaN_3 and stained with Coomassie brilliant blue and destained.

In vitro *invasion assay*

Cellular invasiveness to reconstituted basement membrane–coated filters in the membrane invasion culture system (MICS, modified Boyden chamber) was determined as previously described (Hendrix *et al.,* 1987). Briefly, a pre-cut, gas-sterilized polycarbonate membrane with 8 μm diameter pores was coated with cold Matrigel (100 μg/filter; Becton Dickinson, Bedford, MA), air-dried and positioned between the upper and lower plates of each MICS chamber. Into each upper well were seeded 2×10^5 cells in a random manner. After 72 hr incubation, cells on the upper surface of the filters were completely removed by wiping with a cotton swab, as monitored visually under high power. Filters were fixed in methanol and stained with hematoxylin and eosin. For quantification, cells that migrated to the lower surface were counted under a microscope in 4 pre-determined fields at a magnification of ×200. Assays were also performed with 10 mM 1,10-phenanthroline (Sigma), a metalloproteinase inhibitor.

In vitro *invasion assay of BM314 cells treated with ATRA*

BM314 cells were pre-treated for 3 days with 1 μM ATRA and continuously exposed for an additional 3 days in the *in vitro* invasion assay as described above.

Statistical analysis

Each *in vitro* invasion assay was performed in triplicate. Statistical significance of differences was determined by Student's two-tailed *t* test.

RESULTS

In vitro *invasion assay of colon cancer cells transfected with matrilysin*

We chose CHC-Y1 and DLD-1 colon cancer cells for introduction of matrilysin cDNA because they expressed matrilysin mRNA at an undetectable level by reverse transcription polymerase chain reaction (RT-PCR) (data not shown). The expression of exogenous matrilysin mRNA in colon cancer cells transfected with pcDNAI Mat or pcDNAI Mat-as was examined using Northern blot analysis. Matrilysin mRNA was present in the cells transfected with pcDNAI Mat, indicating that the exogenous matrilysin cDNA was successfully transduced into these cells and efficiently transcribed (Fig. 1*a*). These clones were designated CHC-Mat or DLD-Mat. Matrilysin mRNA was not detected in cells transfected with pcDNAI Mat-as. The expression of MMP-2 mRNA was not affected by transfection of matrilysin cDNA.

The enzyme activity of matrilysin in these cells was examined using casein zymography. Inactive (29 kDa) and activated (19 kDa) forms of matrilysin were observed in culture media

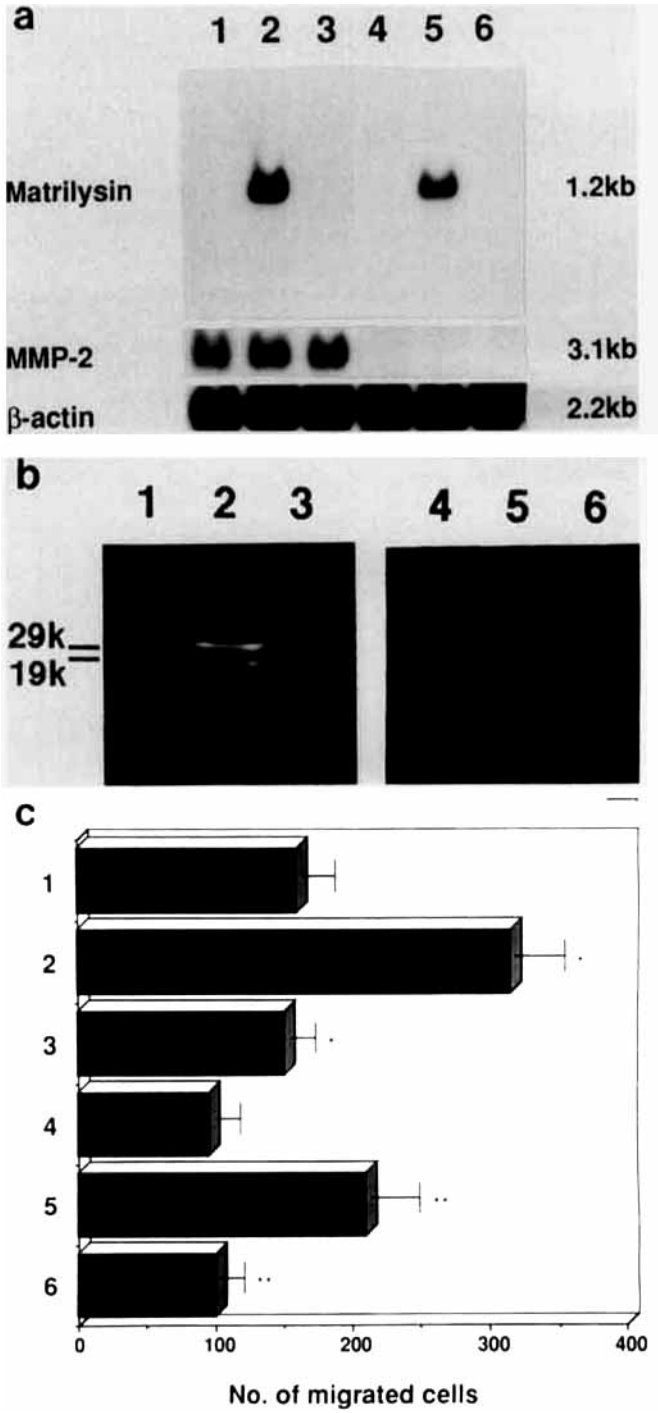

FIGURE 1 – *(a)* Northern blot analysis of transfectants. Ten micrograms of total RNA prepared from each cell were electrophoresed in a 1% agarose/formaldehyde gel. After transfer to a nitrocellulose membrane, blots were hybridized with probes for matrilysin and MMP-2. Blots were stripped and reprobed for β-actin to control for RNA loading and transfer. Analyzed cells are: lane 1, parental CHC-Y1; lane 2, CHC-Mat; lane 3, CHC-co; lane 4, parental DLD-1; lane 5, DLD-Mat; lane 6, DLD-co. *(b)* Zymographic analysis of transfectants. Cell number–adjusted aliquots of the culture media from cells grown for 24 hr in serum-free medium were electrophoresed in a 10% polyacrylamide gel embedded with 1 mg/ml casein. After electrophoresis enzymes were renatured and incubated at 37°C for 18 hr. Proteinases were detected as transparent bands on the blue background of Coomassie blue–stained gels. Numbers on the left are m.w. in thousands. Analyzed cells are the same as *(a)*. *(c) In vitro* invasion assay of transfectants. Cells were tested for their ability to invade reconstituted basement membrane–coated filters in the MICS chamber for 72 hr incubation. Cells that migrated to the lower surface were counted under a microscope in 4 pre-determined fields at a magnification of ×200. Analyzed cells are the same as *(a)*. The average number of three separate experiments is shown with S.D. (bar). * and ** indicate that the difference was statistically significant ($*p < 0.05$, $**p < 0.05$).

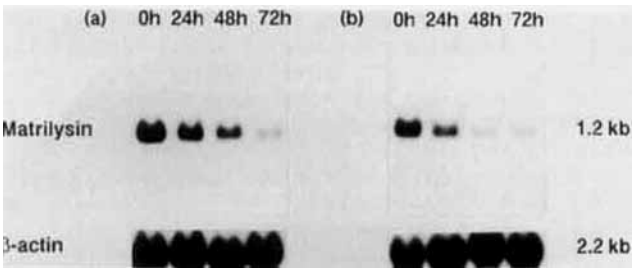

FIGURE 2 – Decreased expression of matrilysin mRNA in BM314 cells treated with TGF-β or with ATRA. BM314 cells were exposed to TGF-β (0.1 ng/ml) or to ATRA (1 μM) for the indicated periods. Total RNA was prepared and analyzed for expression of matrilysin mRNA by Northern blotting as described in Figure 1*a*. TGF-β *(a)*, ATRA *(b)*.

from samples of CHC-Mat or DLD-Mat. Samples of CHC-co, DLD-co or parental cells exhibited no detectable levels of matrilysin (Fig. 1*b*). This result suggests that matrilysin mRNA produced from cells transfected with pcDNAI Mat is efficiently translated into matrilysin protein in these cells. *In vitro* growth rate measured by cell growth kinetics was almost equivalent among these transfectants (data not shown).

These cells were then tested for their potential to invade the filters coated by reconstituted basement membrane in the MICS chamber. CHC-Mat or DLD-Mat demonstrated about twice the invasive ability of each control cell (Fig. 1*c*). The increased invasive potential after introduction of matrilysin was also observed in several clones for each cell type transfected with pcDNAI Mat (data not shown). To confirm that the results can be related to the expression of matrilysin gene and not to other potential distributions of genome through transfection, we then used a metalloproteinase-specific inhibitor in the invasion assay. The difference in invasive potential between matrilysin transfectants and control cells was removed by a metalloproteinase inhibitor (data not shown). This result supports the finding that enhanced invasion of matrilysin transfectants is related to enhanced matrilysin activity.

Cell treatment

BM314 cells were treated with either TGF-β or ATRA. The expression level of matrilysin mRNA gradually decreased after the addition of TGF-β. ATRA also inhibited the expression of matrilysin mRNA (Fig. 2). The suppressive effect of ATRA on matrilysin gene expression was more marked than that of TGF-β.

In vitro *invasion assay of BM314 cells treated with ATRA*

BM314 cells were pre-treated with 1 μM ATRA for 3 days and continuously exposed for an additional 3 days in the *in vitro* invasion assay. Suppression of the enzyme activity of matrilysin in BM314 cells treated with ATRA was demonstrated using casein zymography (Fig. 3*a*). The expression of other related genes, including MMP-2, MMP-9, TIMP-1 and TIMP-2, was not affected by ATRA (data not shown). BM314 cells treated with ATRA demonstrated about one-third the invasive ability of control cells (Fig. 3*b*). The difference in invasive potential between BM314 cells treated with ATRA and control cells was removed by a metalloproteinase inhibitor but not by a serine protease inhibitor or by anti-sera against either plasminogen or urokinase-type plasminogen activator (data not shown).

In vitro *invasion assay of BM314 transfected with anti-sense matrilysin*

PcDNAI Mat-as, the plasmid designed to produce anti-sense matrilysin RNA, was transfected into BM314 cells. An amount of anti-sense matrilysin RNA comparable to mRNAs of moderate abundance was present in the cytoplasm of 2 of 15 BM314 cells transfected with pcDNAI Mat-as (data not shown). These 2 clones were designated BM-as 5 and BM-as 12. A significant reduction in the steady-state abundance of matrilysin mRNA was observed in these 2 clones (Fig. 4*a*). The expression of other related genes, including MMP-2, MMP-9, TIMP-1 and TIMP-2, was not affected by transfection (data not shown). The enzyme activity of matrilysin in the conditioned culture media of these clones was hardly detectable by casein zymography (Fig. 4*b*). *In vitro* growth rate measured by cell growth kinetics was almost equivalent among these transfectants (data not shown).

The *in vitro* invasive potential of these cells was then assayed (Fig. 4*c*). Both BM-as 5 and BM-as 12 demonstrated about one-half the invasive potential of control cells. The BM-as 9 clone, which behaves like the control cells concerning matrilysin mRNA and enzyme production, demonstrated invasive potential equivalent to that of control cells. The difference in invasive potential between matrilysin-reduced BM314 cells and control cells was removed by a metalloproteinase inhibitor (data not shown).

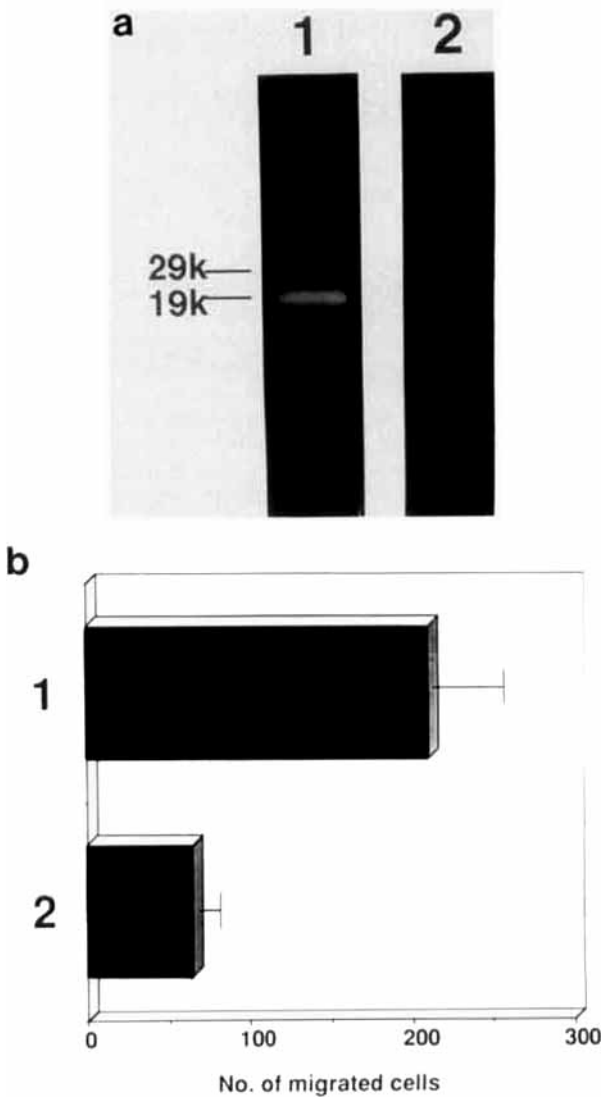

FIGURE 3 – *(a)* Zymographic analysis of BM314 cells treated with ATRA. Assays were performed as described in Figure 1*b*. Analyzed cells are: lane 1, control BM314 cells; lane 2, BM314 cells treated with ATRA. *(b) In vitro* invasion assay of BM314 cells treated with ATRA. Assays were performed as described in Figure 1*c*. The difference observed between BM314 cells treated with ATRA and control cells was statistically significant ($p < 0.05$).

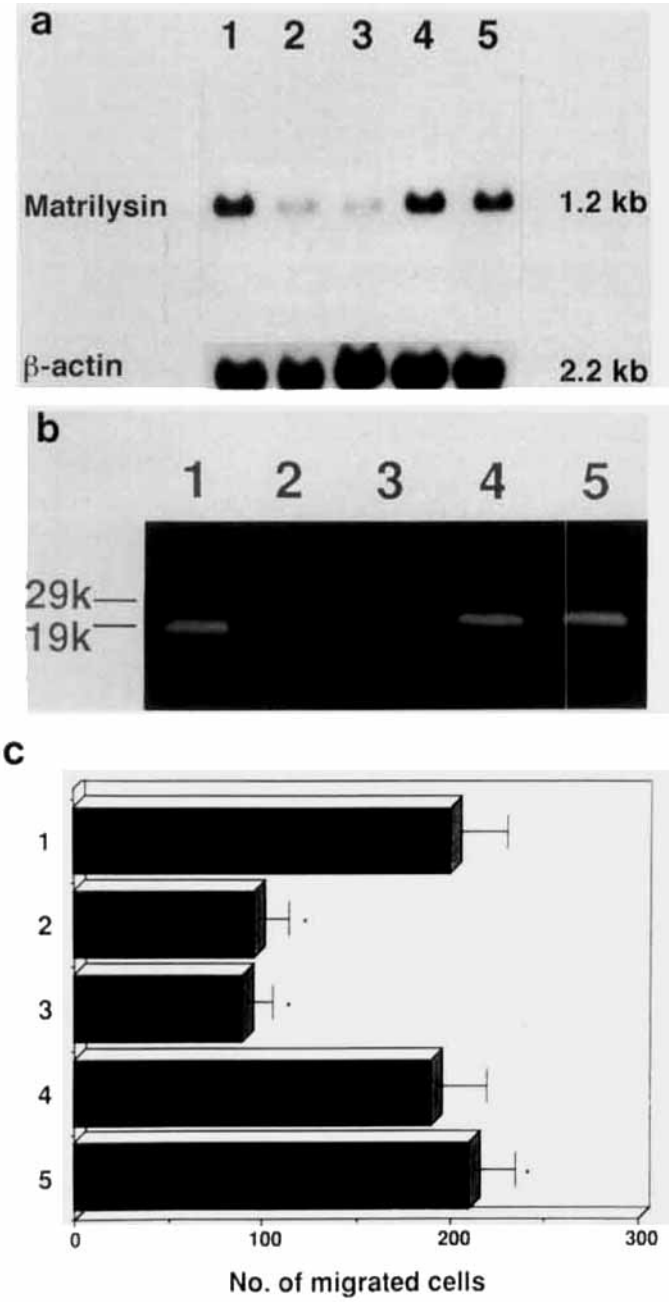

FIGURE 4 – *(a)* Northern blot analysis of BM314 cells transfected with anti-sense matrilysin. Northern blotting was performed as described in Figure 1*a*. Analyzed cells are: lane 1, parental BM314 cells; lane 2, BM-as 5; lane 3, BM-as 12; lane 4, BM-as 9; lane 5, BM-co. *(b)* Zymographic analysis of BM314 cells transfected with anti-sense matrilysin. Assays were performed as described in Figure 1*b*. Analyzed cells are the same as *(a)*. *(c) In vitro* invasion assay of BM314 cells transfected with anti-sense matrilysin. Assays were performed as described in Figure 1*c*. *Difference observed between BM-as 5 or 12 and BM-co was statistically significant ($p < 0.05$). Analyzed cells are the same as *(a)*.

DISCUSSION

We modulated the expression of matrilysin in colon cancer cells to gain insight into the relationship of this metalloproteinase to invasiveness. Zymographic analysis revealed that colon cancer cells transfected with matrilysin cDNA secreted inactive and activated forms of matrilysin. Other proteinases, such as serine protease, heparanase or thiol-dependent enzymes, may be involved in the activation of this enzyme, either in solution or through a cell surface-dependent mechanism (Liotta and Stetler-Stevenson, 1990; Blasi and Verde, 1990). Over-expression of this metalloproteinase rendered these colon cancer cells more invasive, suggesting that matrilysin may contribute to invasion of these colon cancer cells.

Previous studies have shown that matrilysin, like other metalloproteinases, is up-regulated by TPA and EGF (Gaire *et al.*, 1994). However, down-regulation of matrilysin expression has not been reported. Investigating this aspect is important to understand the possible roles of matrilysin in colorectal cancer progression. We have shown that TGF-β or ATRA decreased the expression of matrilysin in BM314 cells, suggesting a possible application of these compounds in the treatment of invasive and metastatic colon cancer. Both compounds suppress the transformed phenotype and enhance differentiation of many types of tumor cell in culture and *in vivo* (Sporn *et al.*, 1987; Nakajima *et al.*, 1989; Hendrix *et al.*, 1990). Therefore, suppression of matrilysin by these compounds may reflect suppression of the poorly differentiated phenotype in colon cancer cells. Furthermore, the matrilysin promoter contains sequences with a high homology to the TGF-β inhibitory element originally identified in the rat stromelysin promoter (Gaire *et al.*, 1994). Therefore, the suppressive effect of TGF-β on matrilysin gene expression in BM314 cells might be regulated by this element.

These results led us to examine the effects of these compounds on the ability of colon cancer cells to migrate across an artificial membrane *in vitro*. However, TGF-β has been shown to suppress the expression of MMP-1 and MMP-3, while it induces MMP-2 and MMP-9 in various cancer cells (Welch *et al.*, 1990). Therefore, ATRA is a more appropriate choice for possible application for the treatment of invasive and metastatic colon cancer. We examined the effects of down-regulation of matrilysin by ATRA on the invasive potential of BM314 cells. As we anticipated, BM314 cells treated with ATRA demonstrated about one-third the invasive potential of control cells. A metalloproteinase inhibitor removed the difference of invasive potential between BM314 cells treated with ATRA and control cells. In contrast, aprotinin or anti-sera against either plasminogen or urokinase-type plasminogen activator did not. The expression of other related genes, including MMP-2, MMP-9, TIMP-1 and TIMP-2, was not affected by ATRA in BM314 cells. This suggests that matrilysin may play a critical role in the invasion of BM314 cells and that the use of ATRA as an anti-metalloproteinase therapy may be effective in the treatment of human colon cancer.

However, we cannot rule out the possibility that ATRA modulates other invasion-related genes in BM314 cells. To clarify this point, we directly down-regulated the expression of matrilysin by the introduction of an anti-sense matrilysin into BM314 cells. These matrilysin-reduced BM314 cells demonstrated about one-half the invasive potential of control cells. In this transfection experiment differences in characteristics among anti-sense matrilysin-transfectants are supposed to be due to factors such as the site of integration of the transfection. However, we have shown that the difference in invasive potential between matrilysin-reduced BM314 cells and control cells was removed by a metalloproteinase inhibitor. This has provided evidence that the reduced invasion of anti-sense matrilysin transfectants is related to down-expression of matrilysin. Together, our results confirm that matrilysin contributes to the invasive phenotype of colon cancer cells.

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