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Advances in Brief

Anti-Invasive Activity of Ursolic Acid Correlates with the Reduced Expression of Matrix Metalloproteinase-9 (MMP-9) in HT1080 Human Fibrosarcoma Cells[1]

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Abstract

We examined the anti-invasive activity of ursolic acid (UA) on the highly metastatic HT1080 human fibrosarcoma cell line. UA reduced tumor cell invasion through a reconstituted basement membrane in a transwell chamber. A significant down-regulation of matrix metalloproteinase-9 [MMP-9; M_r 92,000 gelatinase/type IV collagenase (gelatinase B)] by UA was detected by Northern blot analysis. However, MMP-2 [M_r 72,000 gelatinase/type IV collagenase (gelatinase A)] and membrane-type MMP were constantly expressed, and the expression of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 also was not changed after 3 and 6 days of treatment with UA. Quantitative gelatin-based zymography confirmed a markedly reduced expression of MMP-9 but not MMP-2 after treatment with UA. To confirm the UA-induced down-regulation of MMP-9 expression, we constructed a secreted alkaline phosphatase (SEAP) reporter vector including MMP-9 promoter. After transfection of MMP-9/SEAP reporter vector into HT1080 cells, reduced SEAP activity was detected after treatment with UA. These results suggest that down-regulation of MMP-9 contributes to the anti-invasive activity of UA in HT1080 cells.

Introduction

Invasion into surrounding tissues is a characteristic feature of malignant tumors, and such invasiveness is also required for tumor cells to form metastatic colonies. An essential pattern of this process includes degradation of the ECM[3] and BM. Many proteolytic enzymes produced by tumor cells have been reported to degrade components of the ECM and BM. The MMPs are members of the unique family of proteolytic enzymes which contain a zinc ion at their active sites and can degrade native collagens and other ECM components. Therefore, MMPs are believed to play a role in the invasion of the basement membrane by tumor cells (1). MT-MMP was identified recently in human placenta and proposed to be an activator of MMP-2 (2). The activities of the MMPs are regulated by endogenous proteins known as TIMPs. Since latent forms of MMP-2 and MMP-9 can form 1:1 complexes with TIMP-2 and TIMP-1, respectively, these complexes may also alter sensitivity for activation of the enzymes (3).

UA, pentacyclic triterpene acid, has been isolated from many kinds of medicinal plants, such as *Eriobotrya japonica, Rosmarinus officinalis,* and *Glechoma hederaceae* (4–7). Especially, *Eriobotrya japonica* has been used as an anticancer drug in the traditional herbal medicine of Korea. UA has been reported to produce antitumor activities including inhibition of skin tumorigenesis (4), induction of tumor cell differentiation (5), and antitumor promotion (6, 7). In addition, UA was shown recently to possess an antiangiogenic effect in chick chorioallantoic membrane (8). Taken together, these antitumorigenic activities of UA might be related to the anti-invasive activity on tumor cells.

Therefore, we focused our attention on whether UA reduces the tumor cell invasion *in vitro*. For this purpose, we examined the anti-invasive activity of UA on the highly metastatic HT1080 human fibrosarcoma cell line with an *in vitro* invasion assay. Then we analyzed the expressions of several proteolytic enzymes that were related to tumor invasion. As a result, the down-regulation of the expression of MMP-9 was detected by Northern blot analysis and gelatin-based zymography. In addition, in the SEAP assay using the MMP-9 promoter region, the reduced SEAP activity was also detected after treatment with UA. The data presented show that the anti-invasive effect of UA on the HT1080 cells was caused by the reduced expression of the MMP-9 gene.

Materials and Methods

Cell Culture and Treatment with UA. HT1080 human fibrosarcoma cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. UA was obtained from Sigma Chemical Co. (St. Louis, MO), and the cells were treated with 5, 7.5, or 10 μM of UA. After a 3- or 6-day incubation, the cells were harvested or conditioned media were collected for Northern blot analysis and gelatin-based zymogram assay.

***In Vitro* Invasion Assay.** *In vitro* invasion assay was carried out by the method of Saiki *et al.* (9). Invasion was measured by use of 24-well transwell units with 8 μm porosity polycarbonate filters. The lower side of the filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μl of 0.5 mg/ml reconstituted basement membrane substance (Matrigel; Collaborative Research, Lexington, KY). The coated filters were air-dried for 1 h prior to the addition of the cells. The lower compartment contained 600 μl of DMEM containing 0.1 mg/ml BSA, and 5×10^4 cells were resuspended in 100 μl DMEM and placed in the upper part of a transwell plate. The same concentration of UA as that being cultured was treated in the upper and lower parts of the transwell plate, and cells were incubated for 16 h in a humidified atmosphere of 5% CO_2 at 37°C. Cells were fixed with methanol and stained with H&E. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and invasion was determined by counting the cells that migrated to the lower side of the filter with optical microscopy at ×400. Thirteen fields were counted for each assay. Each sample was assayed in triplicate, and each assay was repeated twice.

RNA Extraction and Northern Blot Analysis. Total cellular RNA was prepared from HT1080 cells according to the acid-guanidinium thiocyanate-phenol-chloroform extraction method (10). The RNA samples were resolved on 1% agarose/formaldehyde gels under denaturing conditions and transferred to nylon membranes (Zeta-Probe membrane; Bio-Rad). The RNA was hybridized to [^{32}P]dCTP-labeled cDNA probes of MMP-2, MMP-9, MT-MMP,

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[3] The abbreviations used are: ECM, extracellular matrix; BM, basement membrane; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; TIMP, tissue inhibitor of metalloproteinase; SEAP, secreted alkaline phosphatase; UA, ursolic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

TIMP-1, and TIMP-2 (11, 12). The filter was prehybridized at 42°C for 4 h in a polyethylene bag with hybridization buffer containing 50% deionized formamide, 7% SDS, 0.12 M $NaHPO_4$, and 0.25 M NaCl and then hybridized at 42°C overnight in hybridization buffer with denatured labeled probes. After hybridization, the filter was washed at 42°C in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS for 15 min and 0.5× SSC, 0.1% SDS for 15 min. The filter was then exposed to X-ray film for 1 day.

Gelatin Zymography. The amount of gelatinase (MMP-2 and MMP-9) in the conditioned media was quantified by cell number. The conditioned media of 10^4 cells were analyzed by gelatin-based zymography, using a slightly modified procedure from that of Herron *et al.* (13). Conditioned media were separated by SDS-PAGE using 10% acrylamide copolymerized with gelatin (0.33 mg/ml). After electrophoresis, the gel was rinsed twice with 2.5% Triton X-100 for 15 min and incubated for 18 h at 37°C in incubation buffer [0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% NaN_3]. Gelatinase was identified following staining of the gel in 0.25% Coomassie blue R250 and destaining in 7% acetic acid. The digested area appeared clear on a blue background, indicating the location of gelatinase.

SEAP and MTT Assay. SEAP reporter vector (pBC12/PL/SEAP) was kindly provided by Dr. M. H. Malim (Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA). SEAP reporter vector containing MMP-9 promoter region (14, 15) was constructed by ligating a 673 bp (−670 to +3) of the MMP-9 promoter region to the cloning site of the SEAP reporter vector. SEAP reporter vector was cotransfected with neomycin-resistant (neo) expression vector into the HT1080 cells by the calcium phosphate method (16) and screened under the G418 for 2 weeks to select stable transfectant cells. SEAP activity was measured by the method described previously (17). Cultured media of the transfected cells after 3- or 6-day treatments with UA, and neomycin-transfected HT1080 cells, as a negative control, were incubated at 65°C for 5 min to inactivate endogenous and serum-contained alkaline phosphatase. Then the media were spun at 15,000 rpm for 5 min, and 100 μl of the media were transferred to a flat-bottomed microtiter plate. One hundred μl of 2× SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, and 20 mM L-homoarginine) were added, and the mixture was incubated for 10 min at 37°C. Twenty μl of 120 mM *p*-nitrophenol phosphate were added in each well, and after 12 h of incubation at 37°C, light absorbance at 405 nm (A_{405}) was measured with an ELISA reader.

The number of viable cells was analyzed by the MTT assay (18). MTT (0.1 mg/ml) was added to each well and incubated at 37°C for 4 h; cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells forms a blue formazan product, which can be measured spectrophotometrically at 540 nm.

Results

Anti-Invasive Activity of UA on HT1080 Cells. We examined the effect of UA on HT1080 cell invasion through a reconstituted basement membrane (Matrigel) in a transwell chamber. The invasion of HT1080 cells through Matrigel to the collagen-coated lower surface of the filters was inhibited by UA in a concentration-dependent manner (Fig. 1). Treatment with 10 μM of UA for 6 days inhibited the invasion of HT1080 cells into Matrigel by about 80% compared with a control.

Reduced Expression of MMP-9 Gene by UA. To elucidate the mechanism of anti-invasive action of UA, we investigated the effect of UA on the expression of MMPs and TIMPs by Northern blot analysis. As shown in Fig. 2, treatment of HT1080 cells with UA for 6 days significantly reduced the expression of MMP-9 in a concentration-dependent manner, whereas the 3-day treatment with UA did not affect the expression of MMP-9. Moreover, the expression of MMP-2, MT-MMP, TIMP-1, and TIMP-2 was not significantly changed by UA treatment. MMP-1 and MMP-3 were also analyzed by Northern blot analysis but were not detected because their basal level expressions were extremely low (data not shown). Similar results were obtained when the level of each protein was measured by gelatin-based zymography (Fig. 3). The amount and activity of MMP-9 protein (M_r 92,000) in the conditioned medium from the cells

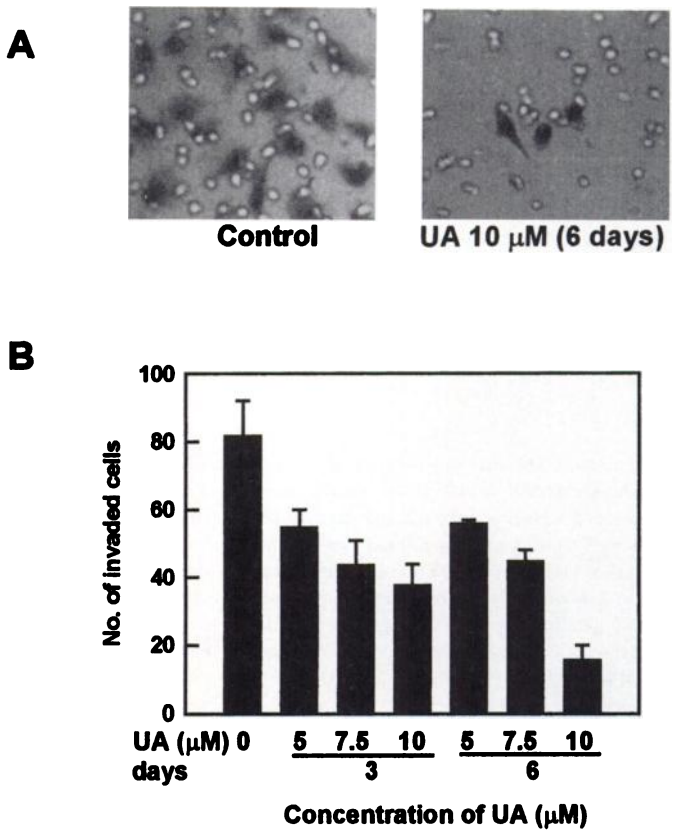

Fig. 1. *In vitro* invasion assay of HT1080 cells after treatment with UA. *A*, light microscopic examination of UA-untreated HT1080 cells (*left*) and 10 μM HT1080 cells after 6 days of treatment (*right*) (×200). *B*, anti-invasive activity of UA. After treatment with 5, 7.5, or 10 μM UA for 3 or 6 days, the cultured HT1080 cells were incubated in a transwell chamber for 16 h. The number of invaded cells was counted, and mean values were determined under ×400 light microscopy.

treated with 10 μM UA for 6 days were remarkably lower than those of the untreated cells. In accordance with Northern blotting data, the quantity and activity of MMP-2 protein (M_r 72,000) were not significantly changed by the UA treatment (Fig. 3).

Inhibition of Promoter Activity of MMP-9 by UA. The possibility of the action of UA on the MMP-9 promoter activity was confirmed by the SEAP reporter vector containing MMP-9 promoter. The SEAP reporter vector was cotransfected into HT1080 cells with the neo expression vector, and stable transfectant cells were selected. Neo expression vector alone was also transfected, and the transfectant cells were used as a negative control. The same number of cells was seeded, and the relative SEAP activity of conditioned medium from the cells was measured after treatment with UA for 3 or 6 days. The SEAP activity decreased markedly after treatment with 7.5 and 10 μM UA for 3 and 6 days (Fig. 4*A*), but 5 μM UA did not affect the promoter activity of MMP-9 at 3 days of treatment. Since the SEAP activity could be dependent on the cell number, we measured the number of viable cells by MTT assay. Even in the treatment with UA, the viable cell number was not changed markedly, as shown in Fig. 4*B*.

Discussion

Metastasis is a multistep process that involves invasion in a number of sequential steps: invasion through BM, intravasation, extravasation, metastatic invasion into the tissues of distant organs, and again intravasation to start another metastatic cascade (1). In this cascade of metastasis, the invasion of BM by tumor cells is thought to be one of the critical steps. Indeed, the ability of tumor cells to degrade com-

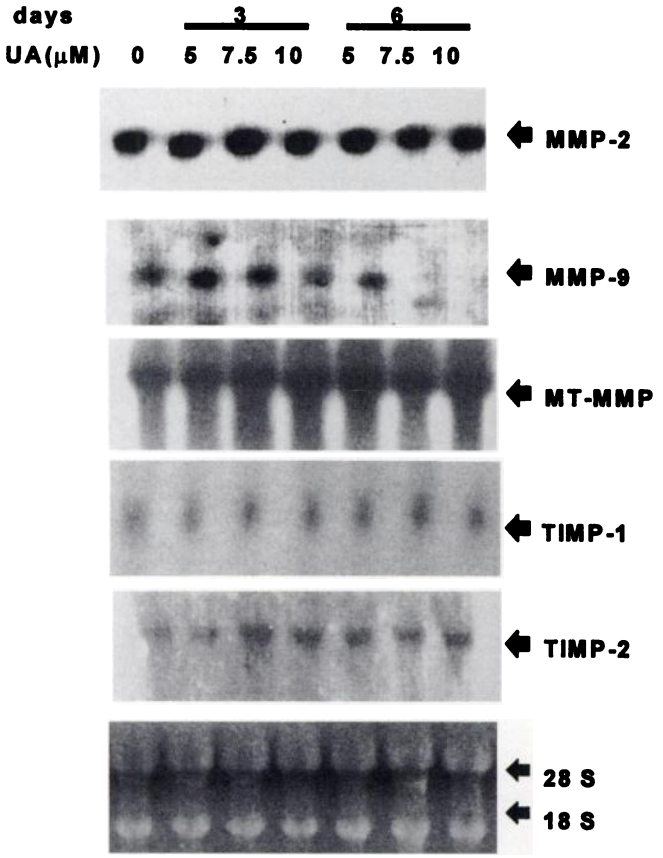

Fig. 2. Northern blot analysis of MMP-2, MMP-9, MT-MMP, TIMP-1, and TIMP-2 in HT1080 cells after treatment with UA. HT1080 cells were treated with 5, 7.5, or 10 μM UA and cultured for 3 or 6 days. Northern blots were hybridized with ^{32}P-labeled cDNA probes of MMP-2, MMP-9, MT-MMP, TIMP-1, and TIMP-2. Molecular sizes of the transcripts are 3.1, 2.8, 4.5, 0.9, and 3.5 kb for MMP-2, MMP-9, MT-MMP, TIMP-1, and TIMP-2, respectively.

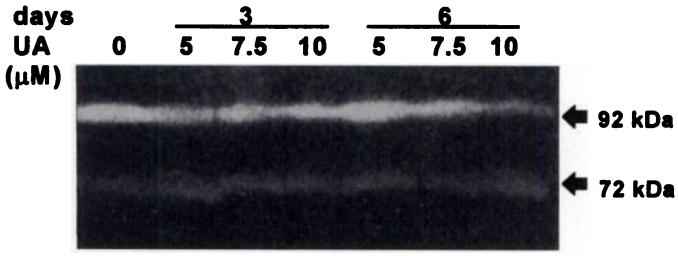

Fig. 3. Gelatin-based zymography of the culture medium of HT1080 cells treated with UA. After treatment with 5, 7.5, or 10 μM UA for 3 or 6 days, the culture media were used in gelatin-based electrophoresis and stained with Coomassie brilliant blue. *Arrows*, the M_r 92,000 and M_r 72,000 type IV collagenases.

ponents of BM has been reported to be correlated with the metastatic potential of cells (19). The BM consists of types IV and V collagens, laminin, other specific glycoproteins, and heparin sulfate proteoglycans. Thus, MMP may be of particular importance in the invasion through these ECM proteins (1). Among these metalloproteinases, a strong correlation between production of type IV collagenase and metastasis has been shown for some transformed cell lines (19, 20).

The present study shows that UA has an inhibitory effect on the invasive activity of the HT1080 cells in a concentration-dependent manner, suggesting the possibility that UA has a capability to inhibit tumor cell invasion. Invasion of the basement membrane by tumor cells is mediated by many proteolytic enzymes including MMPs. The activities of MMPs are regulated by the TIMPs. To identify the anti-invasive mechanism of UA, we have investigated the mRNA expression of MMPs and TIMPs. The results of Northern blot analysis showed that the mRNA expression of MT-MMP, MMP-2, TIMP-1, and TIMP-2 was not changed significantly, whereas the expression of mRNA for the MMP-9 was reduced by UA in a concentration-dependent manner. These results suggest that the inhibitory effect of UA on tumor cell invasion can be partially attributable to the down-regulation of the expression of MMP-9. To confirm that the decreased mRNA level of MMP-9 in the UA-treated HT1080 cells resulted in inhibition of matrix degradation, we examined matrix metalloproteinase activity in HT1080 cells treated with UA by gelatin-based zymography. Two bands of gelatinolysis, M_r 92,000 and M_r 72,000, were detected in the conditioned media from HT1080 cells. The gelatinolytic activity of M_r 92,000 MMP-9 was remarkably down-regulated in the UA-treated HT1080 cells, whereas that of M_r 72,000 was almost the same. These results are in concert with the results of Northern blot analysis and suggest that the decreased transcription of the MMP-9 gene resulted in the down-regulation of MMP-9 activity. In addition, promoter activity of the MMP-9 was also analyzed by the SEAP assay. Relative SEAP activity of the UA-treated HT1080 cells was markedly low in comparison with that of the untreated HT1080 cells. This result indicates that UA acts on the promoter of MMP-9 and down-regulates the expression of the MMP-9. In conclusion, we have demonstrated that UA inhibits tumor cell invasion *in vitro* by inhibiting the transcription of the MMP-9 gene required for the degradation of BM.

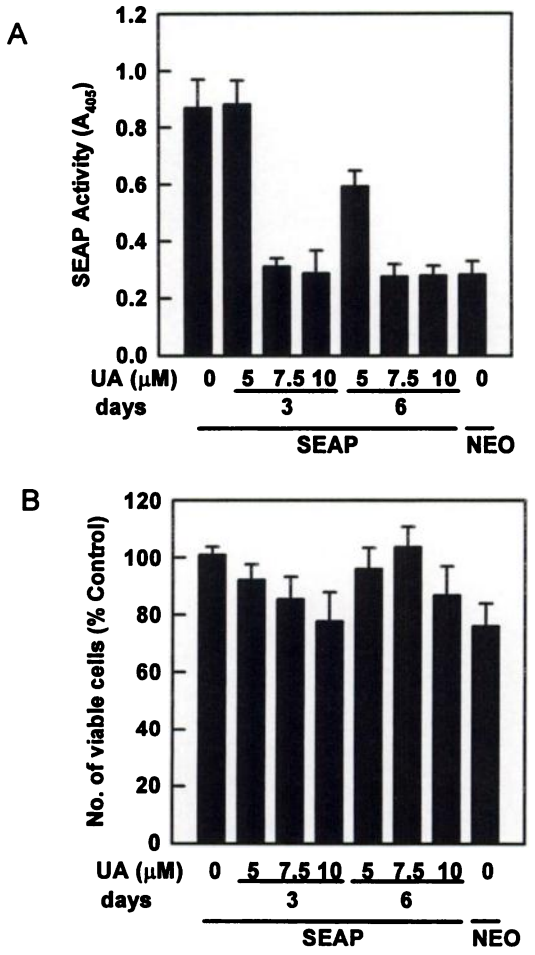

Fig. 4. SEAP activity and MTT assay of UA-treated and untreated HT1080 cells. *A*, SEAP activity of the UA-treated HT1080 cells. The SEAP activity was measured after 3 or 6 days of treatment with UA in the SEAP-transfected and neo-transfected (control) HT1080 cells. *B*, MTT assay of HT1080 cells treated with UA. The number of viable cells was measured by MTT assay after 3 or 6 days of treatment with UA. This assay was used as a control experiment for the number of viable cells after treatment with UA.

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