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Ursolic acid-induced down-regulation of MMP-9 gene is mediated through the nuclear translocation of glucocorticoid receptor in HT1080 human fibrosarcoma cells

Hee-Jae Cha[1,2], Moon-Taek Park[2], Hae-Young Chung[3], Nam Deuk Kim[3], Hiroshi Sato[4], Motoharu Seiki[5] and Kyu-Won Kim[2]

[1]Research Institute of Basic Sciences; [2]Department of Molecular Biology and Pusan Cancer Research Center; [3]Department of Pharmacy, Pusan National University, Pusan 609-735, Korea; [4]Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920; [5]Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, 4-6-1 Shirogane-dai, Minato-ku, Tokyo 108, Japan

We have previously reported that ursolic acid, a pentacyclic triterpene acid, inhibited the invasion of HT1080 human fibrosarcoma cells by reducing the expression of matrix metalloproteinase-9. Since the chemical structure of ursolic acid is very similar to that of dexamethasone, a synthetic glucocorticoid, we investigated whether ursolic acid acts through the glucocorticoid receptor. The expression of matrix metalloproteinase-9 is thought to be regulated similarly with matrix metalloproteinase-1 and matrix metalloproteinase-3 as containing common 2-*O*-tetradecanoylphorbol-acetate responsible region, where AP-1 proteins can bind. Dexamethasone has been studied to repress the 2-*O*-tetradecanoylphorbol-acetate-induced expression of matrix metalloproteinase-1 and matrix metalloproteinase-3 through a glucocorticoid receptor-mediated manner. In Northern blot analysis, we found that ursolic acid reduced the expression of matrix metalloproteinase-1 and matrix metalloproteinase-3 induced by 2-*O*-tetradecanoylphorbol-acetate. Similarly, ursolic acid down-regulated 2-*O*-tetradecanoylphorbol-acetate-induction of matrix metalloproteinase-9 gene in the same manner of dexamethasone. RU486, a potent glucocorticoid receptor antagonist, was used for identifying that ursolic acid-induced down-regulation of matrix metalloproteinase-9 expression is mediated by its binding to glucocorticoid receptor. The effect of ursolic acid on the matrix metalloproteinase-9 expression was blocked by RU486, suggesting that ursolic acid acts via a glucocorticoid receptor in the regulation of matrix metalloproteinase-9. Western blot analysis and immunocytochemistry showed that ursolic acid increased glucocorticoid receptor fraction in the nucleus, although it decreased the synthesis of glucocorticoid receptor mRNA. In addition, ursolic acid did not decrease the expression of c-*jun* and DNA-binding activity of AP-1 to its cognate sequences. Taken together, we suggest that ursolic acid may induce the repression of matrix metalloproteinase-9 by stimulating the nuclear translocation of glucocorticoid receptor, and the translocated glucocorticoid receptor probably down-modulating the *trans*-activating function of AP-1 to 2-*O*-tetradecanoylphorbol-acetate responsible element of matrix metalloproteinase-9 promoter region.

Keywords: ursolic acid; matrix metalloproteinase; glucocorticoid receptor

Correspondence: K-W Kim
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Introduction

Ursolic acid (UA), a pentacyclic triterpene acid, was isolated from many kinds of medicinal plants, such as *Eriobotrya japonica*, *Rosmarinus officinalis*, and *Glechoma hederaceae* (Young *et al*., 1995; Huang *et al*., 1994; Tokuda *et al*., 1986; Ohigashi *et al*., 1986). UA has been reported to produce anti-tumor activities including inhibition of skin tumorigenesis (Huang *et al*., 1994), induction of tumor cell differentiation (Lee *et al*., 1994), anti-tumor promotion (Tokuda *et al*., 1986; Ohigashi *et al*., 1986), and anti-angiogenic effect in chick chorioallantoic membrane (CAM) (Sohn *et al*., 1995). In addition, we previously reported that UA has the anti-invasive activity in the HT1080 human fibrosarcoma cell by reducing the expression of matrix metalloproteinase-9 (MMP-9) (Cha *et al*., 1996).

The promoter region of MMP-9 was characterized as having three important *cis*-element. These elements are possible binding sites for Ap-1, Sp-1, and NF-κB, or related proteins. Deletion or mutation analysis revealed that these three motifs contributed positively to MMP-9 induction by 2-*O*-tetradecanoylphorbol-acetate (TPA) or tumor necrosis factor-α (TNF-α). The AP-1 binding site on MMP-9 gene was shown to play a master role in the induction of MMP-9, cooperating synergistically with the element of the Sp-1-like and the NF-κB-like factors (Sato and Seiki, 1993). The signal to the AP-1 sites is common for the genes of TPA-inducible matrix metalloproteinases such as MMP-1 and MMP-3.

Glucocorticoid hormones have been reported to be potent inhibitors of MMP-1 and MMP-3 of which expression can be induced by AP-1 stimulated by phorbol esters such as TPA (Jonat *et al*., 1990; Nicolson *et al*., 1990; Schüle *et al*., 1990). It has been also reported that the repression of AP-1-responsive genes which is located in a TPA responsible element (TRE) is mediated by glucocorticoid receptor (GR) through a direct interaction with c-Jun (Diamond *et al*., 1990; Lucibello *et al*., 1990; Schüle *et al*., 1990; Touray *et al*., 1991; Yang-Yen *et al*., 1990) or c-Fos (Kerppola *et al*., 1993). However, glucocorticoid does not induce protein synthesis or affect the binding activity of AP-1 to DNA. In fact, the down-modulation of the *trans*-activating function of AP-1 by GR was suggested to be mediated through the interaction with either the pre-existing unbound or DNA-bound AP-1. This suggestion was supported by coprecipitation experiments with AP-1 and GR (Jonat *et al*., 1990).

In this study, we investigated whether UA reduced the expression of MMP-9 through the GR-mediated transrepression of AP-1 since UA has a chemical structure resembling that of dexamethasone (Dexa) (Sohn *et al*., 1995).

Results

Down-regulation of the TPA-induced expression of MMP-1 and MMP-3 genes by UA

GR-mediated transrepression of AP-1-induced genes containing TRE has been completely studied in MMP-1 and MMP-3 (Jonat *et al*., 1990; Nicolson *et al*., 1990; Schüle *et al*., 1990). To identify whether the action of UA is through a GR-mediated manner, we first analysed the effect of UA on the expression of MMP-1 and MMP-3. Since the basal levels of MMP-1 and MMP-3 were extremely low in the HT1080 human fibrosarcoma cells, we induced the level of MMP-1 and MMP-3 by TPA. As demonstrated in Figure 1, treatment with 10 μM of Dexa for 3 or 6 days drastically reduced TPA-induced expression of MMP-1 and MMP-3. Similarly, treatment with 5, 7.5 or 10 μM of UA for 3 or 6 days clearly revealed down-regulation of MMP-1 and MMP-3. From these results, UA clearly reduced the level of MMP-1 and MMP-3 induced by TPA and this pattern was rather milder than the result from Dexa but appeared in good accordance with each other. These data suggested that the action of UA on the transrepression of MMP-1 and MMP-3 might be through the similar mechanism to Dexa.

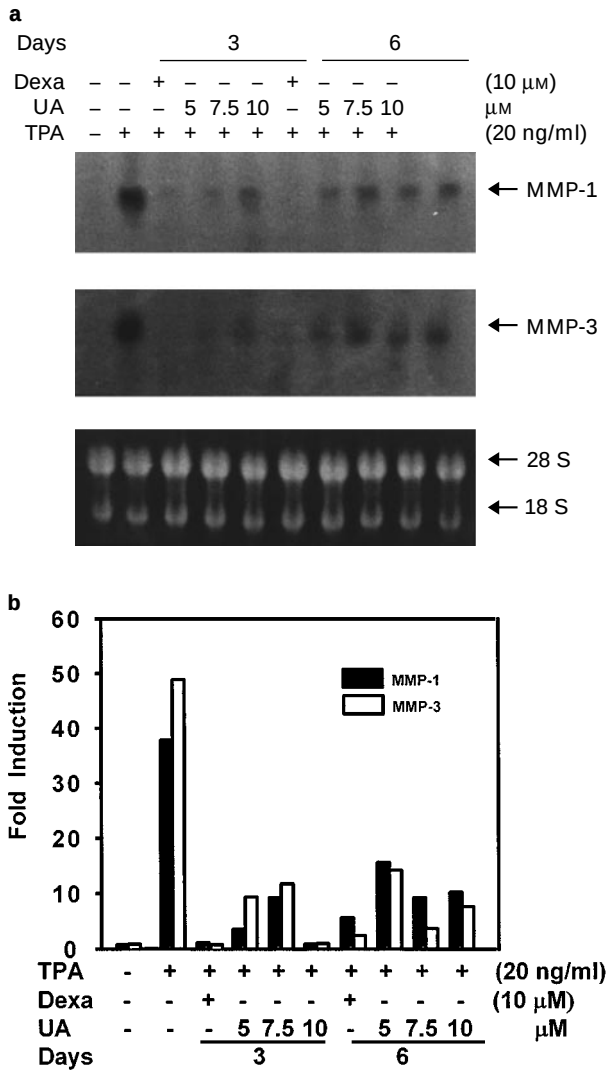

Figure 1 Effects of UA and Dexa on the expression of MMP-1 and MMP-3 induced by TPA. (**a**) HT1080 cells were treated with 5, 7.5 or 10 μM of UA and 10 μM of Dexa for 3 or 6 days. Aliquot of TPA (20 ng/ml) was added 12 h before harvesting the cells. Expression of MMP-1 and MMP-3 was assayed by Northern blot analysis. Filters were hybridized with ^{32}P-labeled cDNA probes of MMP-1, MMP-3. (**b**) The data in (**a**) were quantitated using the Image Pro-Plus (Media Cybernetics) and adjusted to variation in 18S and 28S rRNA

Down-regulation of the TPA-induced expression of MMP-9 gene by UA

We previously reported that UA inhibited the invasion of HT1080 cells by reducing the basal level of MMP-9 expression (Cha *et al*., 1996). We further tried to investigate whether UA can also down-regulate the TPA-induced expression of MMP-9 in the similar manner to those of MMP-1 and MMP-3. Indeed, the expression of MMP-9 was highly induced by TPA and this induction of MMP-9 was reduced by the treatment with 10 μM of Dexa for 6 days about 80% (Figure 2). The expression of TPA-induced MMP-9 was not changed significantly by the treatment of UA for 1

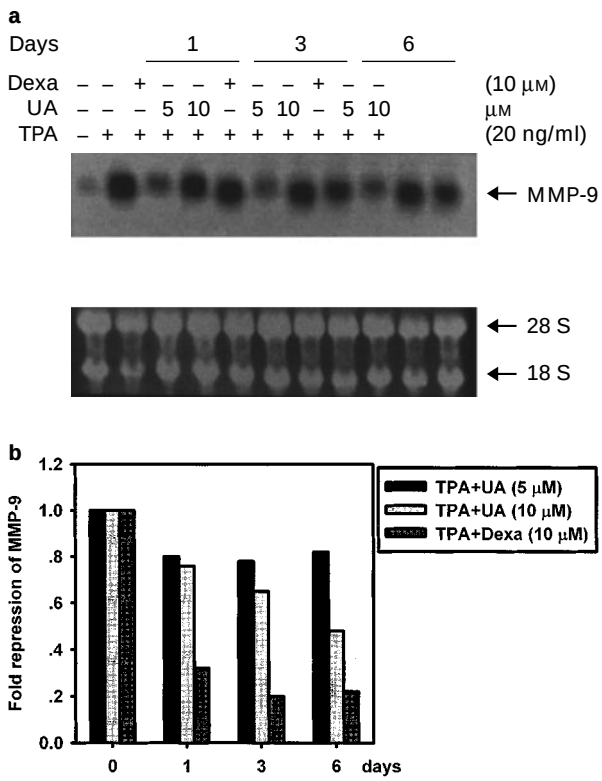

Figure 2 Effects of UA and Dexa on the expression of MMP-9 induced by TPA. (**a**) HT1080 cells were treated with 5 or 10 μM of UA and 10 μM of Dexa for 1, 3 or 6 days. Aliquot of TPA (20 ng/ml) was added 12 h before harvesting the cells. Expression of MMP-9 was assayed by Northern blot analysis. Filters were hybridized with ^{32}P-labeled cDNA probes of MMP-9. (**b**) The data in (**a**) were quantitated using the Image Pro-Plus (Media Cybernetics) and adjusted to variation in 18S and 28S rRNA

day but slightly changed for 3 days, while treatment with UA for 6 days reduced the level of TPA-induced MMP-9 in a concentration-dependent manner up to approximately 50%. Moreover, the effect of UA on the repression of the TPA-induced MMP-9 was confirmed at the protein level by the gelatin-based zymography. As expected, treatment with 10 μM of UA for 6 days reduced the enzyme activity of 92 kDa collagenase (MMP-9) induced by 50%, meanwhile, Dexa down-regulated the enzyme activity by about 90% in the same condition (Figure 3). It is almost the same pattern with the result of Northern blot analysis demonstrated in Figure 2. From the observation of gelatin-based zymography, the level of pro-MMP-2, 72 kDa latent form of type IV collagenase was reduced by TPA. However, either UA or Dexa did not change the activity of pro-MMP-2 (Figure 3a). The activation of 72 kDa pro-MMP-2 to active form (62 kDa) was induced by TPA, which is in accordance with the previous result of Brown *et al*. (1990). Interestingly, this activation was inhibited by treatment with UA or Dexa for 6 days. It shows that UA as well as Dexa probably block the action of TPA. Difference in the ratio of 72 and 92 kDa activities in the first lane between the lower and upper portions of Figure 3a was probably due to the serum containing MMP-2. Therefore, the relative activities of 72 kDa and 92 kDa can be changed according to the number of the cells and the amount of cell culture medium containing 72 kDa enzyme.

From the Northern blot analysis, we confirmed that the mRNA level of MMP-2 was down-regulated by TPA but not changed significantly by either UA or Dexa (Figure 4). It seems, however, that mRNA expression of membrane type-MMP (MT-MMP) was increased by the treatment with UA. It remains unclear what roles of UA plays in the expression of MT-MMP in the TPA-treated HT1080 cells and needs further investigation. The mRNA level of tissue inhibitor of metalloproteinase (TIMP)-2 were not changed by TPA, Dexa or UA. However, the expression of TIMP-1 was markedly induced by TPA but not changed by either Dexa or UA. According to the results of Figures 2, 3 and 4, it is possible to postulate that the specific down-regulation of MMP-9 by UA may have occurred through the similar pathway to MMP-1 and MMP-3.

Effect of RU486 on the down-regulation of MMP-9 by UA

In order to examine the possibility that GR is involved in the down-regulation of 92 kDa MMP-9 by UA, we treated RU486, a potent GR antagonist (Alexandrova, 1992; McRae, 1994). In the gelatin-based zymography, treatment with 10 μM of UA for 6 days reduced the basal level of 92 kDa MMP-9 by 80%, while co-treatment with 10 μM of RU486 for the same periods restored the level of MMP-9 up to approximately 70% of control level (Figure 5a and b). In Figure 5c and d, RU486 clearly recovered the Dexa-induced decreased

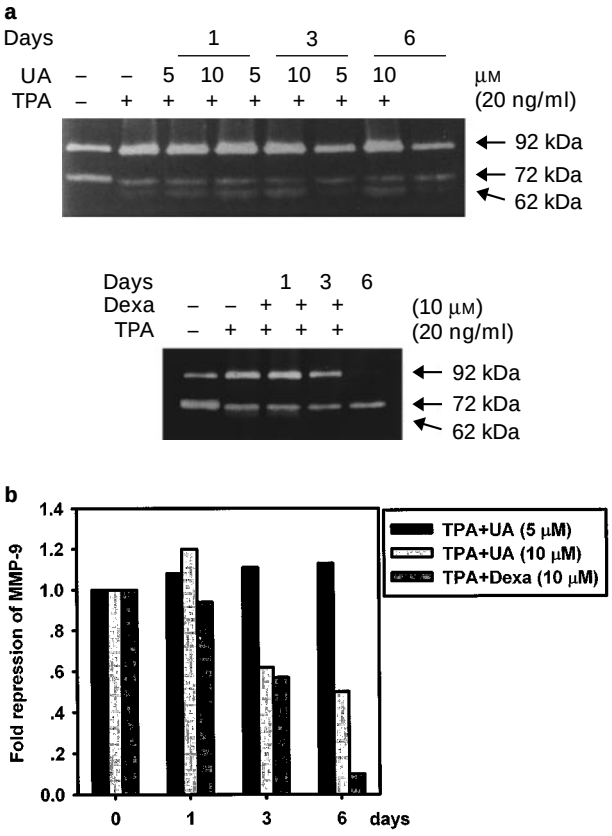

Figure 3 Gelatin based zymography of the culture medium of HT1080 cells treated with UA, Dexa and TPA. (**a**) After treatment with 5 or 10 μM of UA and 10 μM of Dexa for 1, 3 or 6 days and 20 ng/ml TPA for 12 h, the culture media were used in gelatin based electrophoresis and stained with Coomassie brilliant blue. (**b**) The zymogram activity was measured by quantification of bands with the Image Pro-Plus. Arrows indicate the 92 kDa, 72 kDa type IV collagenases, and 62 kDa activated form of 72 kDa type IV collagenase, respectively

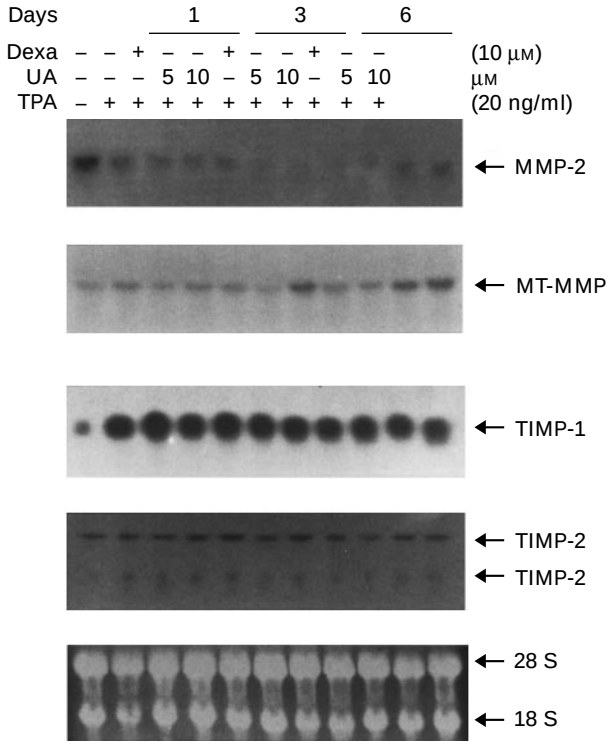

Figure 4 Effects of Dexa and UA on the expression of MMP-2, MT-MMP, TIMP-1 and TIMP-2 in the co-treatment with TPA. HT1080 cells were treated with 5 or 10 μM of UA and 10 μM of Dexa for 1, 3 or 6 days. Aliquot of TPA (20 ng/ml) was added 12 h before harvesting the cells. Expressions of MMP-2, MT-MMP, TIMP-1 and TIMP-2 were assayed by Northern blots. Filters were hybridized with ^{32}P-labeled cDNA probes of MMP-2, MT-MMP, TIMP-1 and TIMP-2

basal level of MMP-9 up to 90% of control. RU486, therefore, clearly blocked down-regulation of MMP-9 by UA or Dexa at the protein level. These results indicate that the action of UA on the down-regulation of MMP-9 might be mediated through the GR.

UA-induced translocation of GR to the nucleus

To determine whether UA induce the translocation of GR from cytosol to nucleus like the action of Dexa, we stained HT1080 cells with anti-GR antibody after treatment with 10 μM of UA for 3 or 6 days. Figure 6a shows that the immunoreactivity of GR in the untreated cells was mainly localized in the cytoplasm whereas treatment with 10 μM of Dexa or UA for 3 days drastically promoted translocation of GR from cytosol to nucleus. However, treatment with UA or Dexa for 6 days diminished the level of translocated GR in the nucleus (data not shown). This result may be caused partly by down-regulation of GR expression by UA or Dexa as previously reported in Rosewicz *et al*. (1988).

To confirm whether UA stimulated the translocation of GR, the nuclear and cytosol extracts were prepared separately for Western blot analysis. Figure 6b indicates that the treatment with UA for 3 days increased the amount of nuclear GR by more than 1.5-fold. In contrast, co-treatment of RU486 with UA for 3 or 6 days potently inhibited the UA-induced translocation of GR to almost same level as control. It seems most likely that UA actually induced translocation of GR into nucleus, which is blocked by the GR antagonist, RU486. Treatment with UA alone or in combination with RU486 for 6 days reduced the level of GR in the nucleus to same level as control and to about 0.8-fold of control, respectively. It seems to be resulted from the down-regulation of GR by UA as referred above. However, we could not detect the difference of the GR level in the cytosolic fraction between UA-treated and untreated cells by Western blot suggesting that the level of GR in the cytosol was much higher compared to that of GR in the nucleus (data not shown).

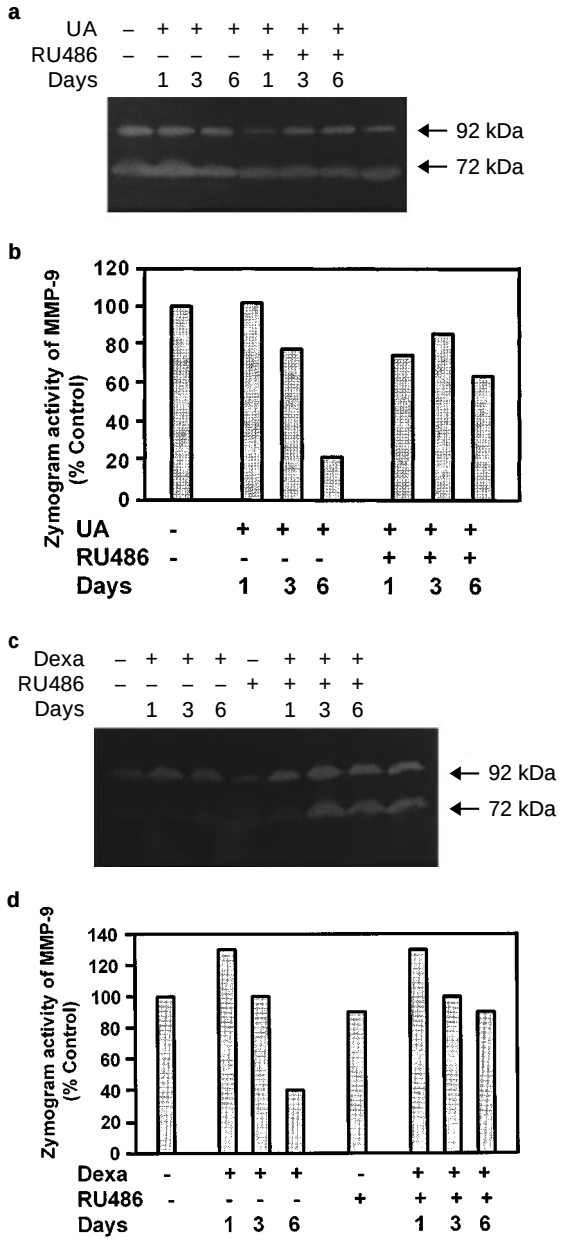

Figure 5 RU486 recovers the down-regulation of MMP-9 expression by UA and Dexa. (**a**) After treatment with 10 μM of UA or 10 μM of RU486 for 1, 3 or 6 days, the culture media of the cells were electrophoresed on the gelatin based zymography. (**b**) The zymogram activity was measured by quantification of bands with the Image Pro-Plus (Media Cybernetics). (**c** and **d**) After treatment with 10 μM of Dexa or 10 μM of RU486 for 1, 3 or 6 days, the culture media were used in gelatin based electrophoresis and stained with Coomassie brilliant blue. The quantification was conducted by Image-Pro-Plus. Arrows indicate the 92 kDa (MMP-9) and 72 kDa type IV collagenase (MMP-2), respectively

*Effect of UA on the expression of c-*jun *and GR*

As shown in above results, UA induced the translocation of GR from the cytosol to the nucleus and may interfere the action of AP-1 for the expression of MMP-9. In addition, we examined whether UA modulates the expression of c-*jun*, a major component of AP-1 or GR. The mRNA levels of c-*jun* and GR were analysed by Northern blot analysis after treatment with UA or Dexa. As shown in Figure 7, the expression of c-*jun* was moderately increased by UA or Dexa. The induction of c-*jun* by Dexa was previously reported by Jonat *et al*. (1990). This increase may be due to a feedback mechanism against the action of Dexa or UA. The treatment with 10 μM of Dexa for 1, 3 or 6 days reduced the mRNA level of GR, which is consistent with the data previously reported by Rosewicz *et al*. (1988). Treatment with 10 μM of UA for 6 days significantly reduced the expression of GR although it is milder than the effect of Dexa (Figure 7).

Effect of UA on the binding of AP-1 to AP-1 binding site on TRE

We conducted a gel retardation assay to measure the interaction between AP-1 and its specific DNA recognition sequences. Following incubation of the nuclear extracts with a radiolabeled double-stranded recognition sequences, the bound oligonucleotide was detected by its mobility retardation in a nondenaturing gel. As shown in Figure 8, nuclear extracts containing the translocated GR in response to Dexa or UA showed no significant reduction in binding

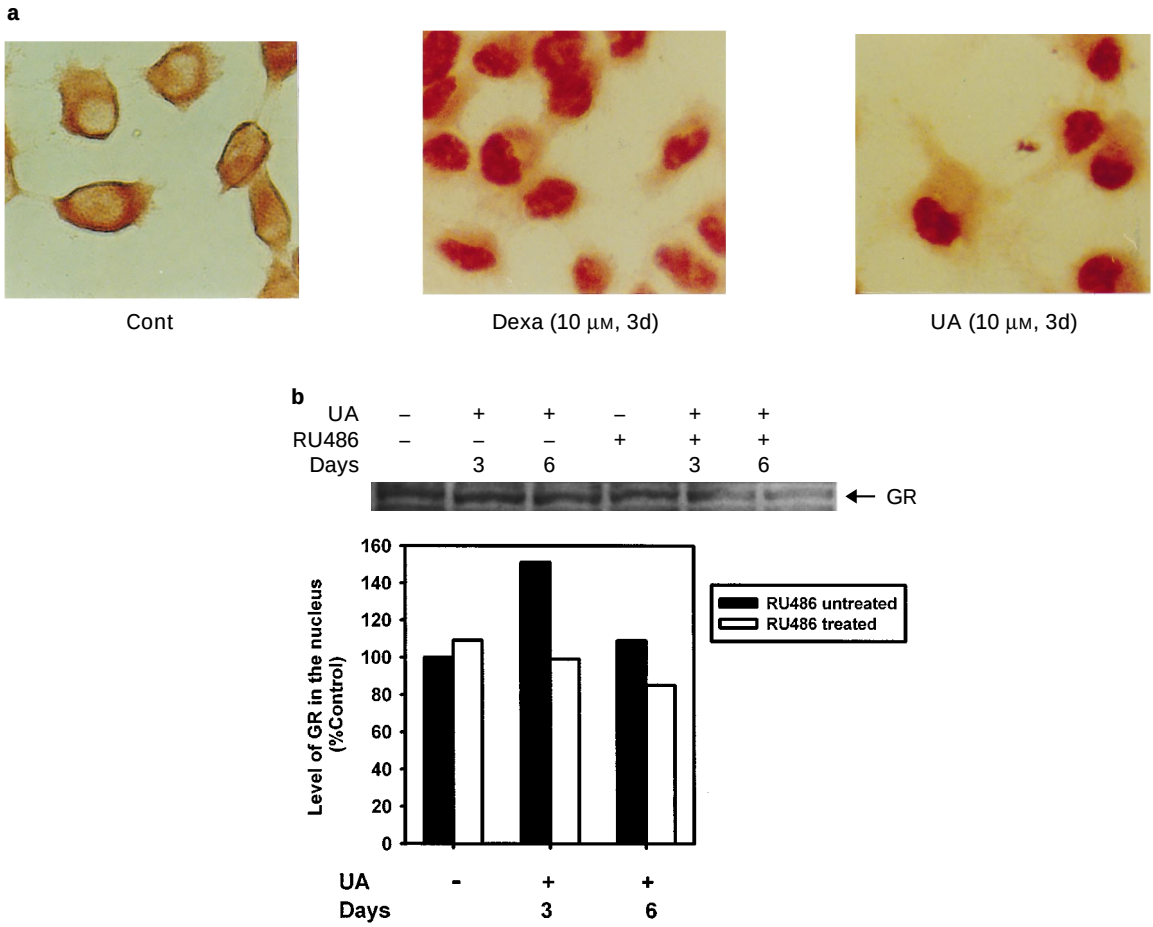

Figure 6 UA induces the nuclear translocation of GR in the immunocytochemistry (**a**) and Western blot analysis (**b**). (**a**) HT1080 cells treated with 10 μM of UA or 10 μM of Dexa for 3 days and immunocytochemical studies were performed as described in Materials and methods. (**b**) After treatment with 10 μM of UA, 10 μM of Dexa or 10 μM of RU486 for 3 or 6 days, the cells were harvested and nuclear proteins (20 μg) were fractionated and transferred to Hybond-ECL. The filters were blotted with anti-GR antibody and detected by ECL and the data were quantitated using the Image-Pro Plus

activity of AP-1 to its binding sequences. On the contrary, UA or Dexa increased the level of AP-1 binding protein. It is consistent with the data about increase of AP-1 binding activity by Dexa, which was reported by Jonat *et al.* (1990). These data may be related to the induction of c-*jun* by UA or Dexa. Therefore, this result suggested that UA-mediated repression of MMP-9 was not mediated through the alteration of binding activity of AP-1 to the cognate sequences on the TRE.

Discussion

It has been previously reported that UA has an inhibitory effect on the invasive activity of the HT1080 cells in a concentration dependent manner (Cha *et al.*, 1996). Invasion of the basement membrane by tumor cells is mediated through many proteolytic enzymes including MMPs (Liotta *et al.*, 1979, 1980; Templeton *et al.*, 1990; Wilhelm *et al.*, 1989; Frederick, 1991). In fact, MMPs have been reported to play a key role in the invasion of tumor cells. We recently demonstrated that the inhibitory effect of UA on tumor cell invasion is partially attributable to the down-regulation of the expression of MMP-9 (Cha *et al.*, 1996).

Since the chemical structure of UA resembles that of Dexa, a synthetic glucocorticoid, we assumed that UA might act as a glucocorticoid through the GR-mediated manner. There have been several mechanisms by which GR-mediated transrepression. These include that GR binds to sequences overlapping a *cis*-acting element for another *trans*-acting factor, thereby displaces it from, or prevents its binding to the cognate element (Akerblom *et al.*, 1988; Drouin *et al.*, 1989; Oro *et al.*, 1988; Strömstedt *et al.*, 1991). GR-mediated transrepression has also been attributed to the direct or indirect interaction of the GR with other *trans*-acting factors, resulting in inhibition of their activity and/or ability to bind to DNA (Celada *et al.*, 1993; Diamond *et al.*, 1990; Gauthier *et al.*, 1993; Jonat *et al.*, 1990; Kutoh *et al.*, 1992; Lucibello *et al.*, 1990; Ray and Prefontaine, 1994; Schüle *et al.*, 1990; Tverberg and Russo, 1992; Yang-Yen *et al.*, 1990). In these model systems, it is necessary that at first, ligand binds with its receptor to stimulate receptor activation, and next, the activated receptor complex be dissociated from hsp90, and then nuclear translocation be occurred. However, the recent report showed that GR-dependent transrepression of AP-1 was ligand-independent, which was observed in transfected cells after heat shock in the absence of ligand (Liu *et al.*, 1995). In particular, the action mechanism of glucocorticoid hormone to inhibit the transcription of collagenases has been suggested by nuclear translocation of GR and its interfering the AP-1, the main

transcriptional activator of collagenases. Dexa activates and translocates GR from cytosol to nucleus by dissociating it from hsp90. The translocated GR inhibits the activity of AP-1 by directly binding to it (Jonat *et al*., 1990; Nicolson *et al*., 1990; Schüle *et al*., 1990; Liu *et al*., 1995).

MMP-9 has the AP-1 binding sequences on the promoter region (Sato *et al*., 1993). Therefore, the decreased expression of MMP-9 by UA could be due to the reduced transactivation activity of AP-1 through the interaction with nuclear GR. Indeed, the TPA-induced MMP-1 and MMP-3 were significantly reduced by Dexa or UA as illustrated in Figure 1. Similarly, Dexa or UA reduced the TPA-induced MMP-9 gene expression (Figure 2). However, TPA-induced MMP-9 was regulated by a rather complicated manner through three major regulatory elements such as NF-κB, Sp-1 binding sites and TRE region (Sato *et al*., 1993). Indeed, it was previously reported that NF-κB and Sp-1 sites are required for TPA-induction of MMP-9 expression (Sato *et al*., 1993). Furthermore, it was reported that TPA is actually involved in the activation of NF-κB (Hirano *et al*., 1995) and inhibitor of NF-κB blocks the action of TPA (Li *et al*., 1997). As shown in Figure 2, UA or Dexa can not completely inhibit the induced expression of MMP-9 by TPA because their major inhibition site is TRE region. Therefore, the effect of UA on MMP-9 is rather milder than those of MMP-1 and MMP-3.

The effects of Dexa and UA on the TPA-induced MMP-9 expression were confirmed by the gelatin-based zymography (Figure 3). In addition, Figure 5 showed that the effect of UA on the reduction of MMP-9 was inhibited by RU486, a potent antagonist of glucocorticoid. These results highly indicate that UA may respond through the GR.

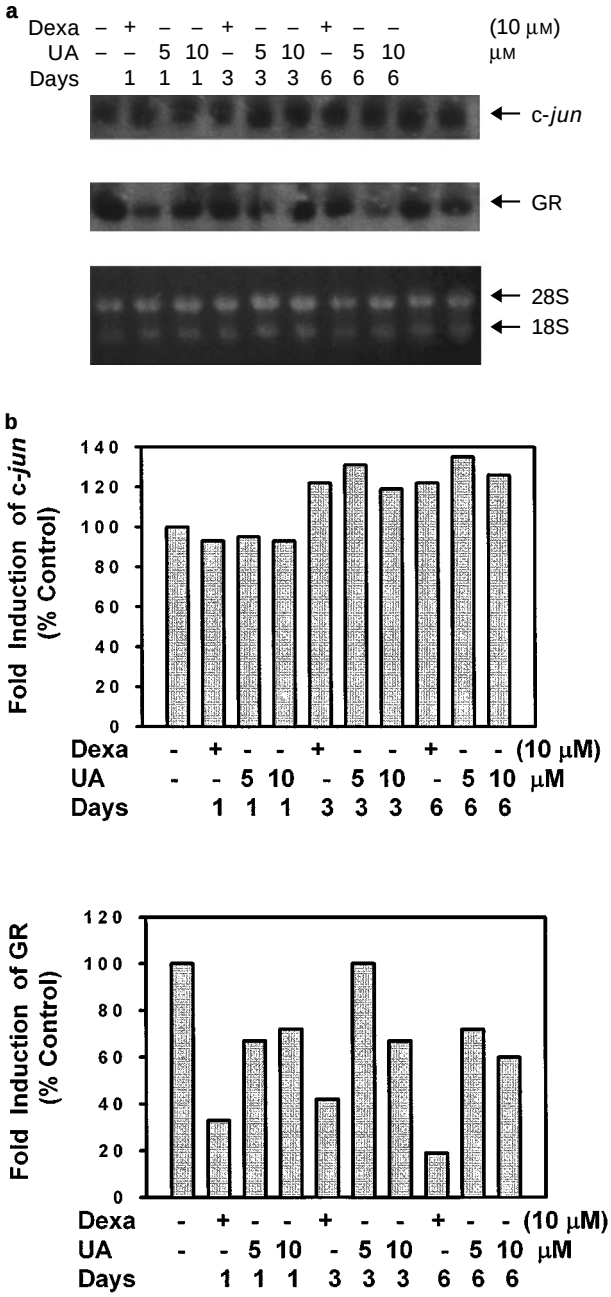

Figure 7 UA does not affect on the expression of c-*jun* and GR. (**a**) HT1080 cells were treated with 5, 10 μM of UA or 10 μM of Dexa for 1, 3 or 6 days. Northern blots were carried out by hybridizing with ^{32}P-labeled cDNA probes of c-*jun* and GR. (**b**) The data in (**a**) were quantitated using the Image Pro-Plus (Media Cybernetics) and adjusted to variation in 18S and 28S rRNA

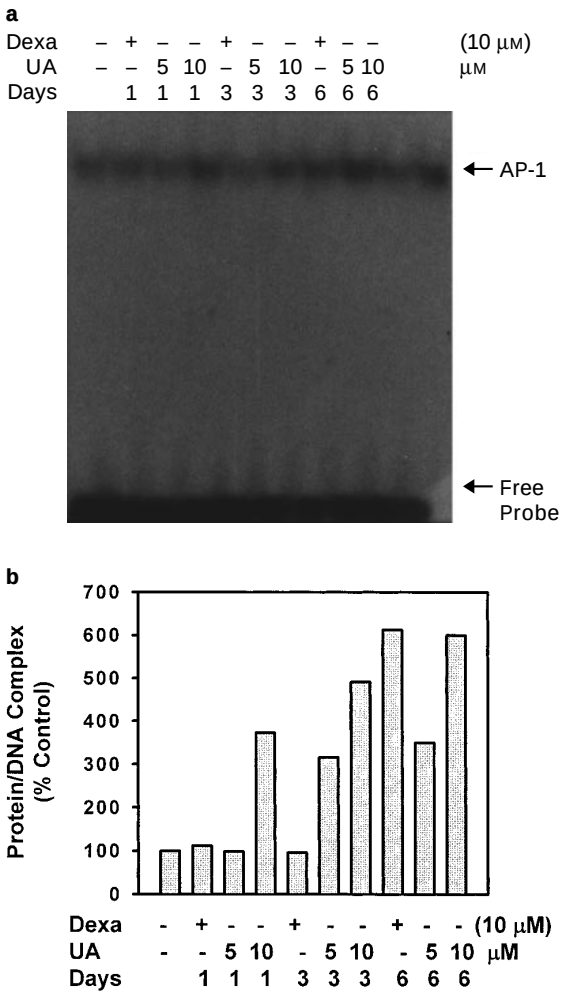

Figure 8 UA does not affect the binding of AP-1 to its binding site on TRE in the gel retardation assay. (**a**) Nuclear extracts (5 μg) from HT1080 cells treated with 5 or 10 μM of UA or 10 μM of Dexa for 1, 3 or 6 days were reacted with a ^{32}P-labeled AP-1 binding oligonucleotides and fractionated on the polyacrylamide gel. (**b**) The data in (**a**) were quantitated using the Image Pro-Plus (Media Cybernetics)

Furthermore, the immunocytochemical analysis demonstrated that treatment with UA for 3 days drastically induced the nuclear translocation of GR and it was confirmed by Western blot analysis of the nuclear and cytosolic fractions, respectively (Figure 6). Previously, we reported that glucocorticoid responsible element (GRE) binding activity was increased by UA in F9 teratocarcinoma cells (Lee *et al*., 1994). It will be another interesting project to further study about UA-mediated transcriptional activation of some target genes, which are activated by GR. UA actually triggered the nuclear translocation of GR, however, the amount of GR in the nucleus was reduced to the same level as control in HT1080 cells treated with UA for 6 days. This reduction may be resulted partly by down-regulation of GR expression by UA as shown in the Figure 7. Thus, we suggest that treatment with UA for 3 days triggered the nuclear translocation of GR maximally and this induction of GR in the nucleus resulted the down-regulation of MMP-9 in HT1080 cells treated with UA for 6 days. However, the level of c-*jun*, which is the main component of AP-1 was increased by UA or Dexa (Figure 7). Dexa was previously reported to increase the expression of c-*jun* and it suggested that UA may act with similar manner to Dexa. In addition, the translocated GR did not inhibit but stimulate the binding activity of AP-1 to the cognate binding site on the TRE in the gel retardation assay (Figure 8). These data suggested the induction of c-*jun* may result in an increase of AP-1 binding and it is consistent with the result of Jonat *et al*. (1990).

Taken together, we suggest that UA may reduce the expression of MMP-9 gene by inhibiting the AP-1 activity through the stimulation of the nuclear translocation of GR.

Materials and methods

Cells and treatment of chemicals

HT1080 human fibrosarcoma cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 μunit/ml penicillin, and 100 μunit/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 10 μM of UA (Sigma Chemical Co., St. Louis), dexamethasone (Sigma Chemical Co., St. Louis), and RU486 for 1, 3 and or 6 days which was kindly provided by Roussel, UCLAF (France). When the cells were treated with TPA, 20 ng/ml of TPA was added for 1 day before harvesting cells.

RNA extraction and Northern blot analysis

Total cellular RNA was prepared from HT1080 cells according to the acid-guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). 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, TIMP-1 and TIMP-2 (Sato *et al*., 1992; Takino *et al*., 1995). 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, 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. Quantitation of bands was routinely performed with Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MA).

Gelatin zymography

The conditioned media of 10^4 cells were analysed by the gelatin-based zymography, using the slightly modified procedure of Herron *et al*. (1986). 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 $ZnCl_2$, 0.02% NaN_3). Gelatinase was identified following staining of the gel in 0.25% Coomassie blue R250 and destaining with 7% acetic acid. The digested area appeared clear on a blue background indicating the location of gelatinase. Zymogram activity was measured by quantification of bands with Image-Pro Plus.

Immunocytochemistry

Immunocytochemistry was conducted as the method of Ylikomi *et al*. (1992). HT1080 cells grown on the coverslip were washed with PBS and incubated in 4% paraformaldehyde for 30 min at room temperature (RT) and washed in PBS. To ensure the entry of antibodies, cells were permeabilized by incubating in 0.5% Triton X-100 for 20 min at RT. After washing with PBS, cells were incubated for 20 min in blocking solution to saturate non-specific sites, followed by incubation with specific GR antibodies for 1 h at RT. After washing with PBS, specifically bound antibodies were detected with biotinylated secondary antibody. After 1 h incubation at RT, cells were washed with PBS and incubated with streptavidin-peroxidase for 30 min at RT. Peroxidase staining was visualized using AEC chromogen as a substrate (DAKO LSAB kit). The immunoperoxidase stained samples were photographed using a phase-contrast microscope (Olympus BX 40).

Western blot analysis

Proteins were separated on 15% non-reducing PAGE (Towin *et al*., 1979). After transfer, nitrocellulose membrane were washed in TBS-T solution (20 mM Tris-HCl buffer, pH 7.6, containing 137 mM NaCl, 1% Tween 20). They were incubated in blocking buffer (5–10% skim milk in TBS-T) for 12 h at 4°C. The filter was incubated with first antibody for 12 h at 4°C, and then washed three times with TBS-T. After washing, the filter was incubated with the second antibody. The band was detected with enhanced chemiluminescence (ECL) reagents according to the supplier's protocol. The quantitation of bands were performed by Image-Pro Plus.

Gel retardation assay

Nuclear extracts were prepared from the slightly modified method of Dignam *et al*. (1983). The cells were harvested, resuspended in 100 μl of lysis buffer (10 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP40, and 0.5 mM PMSF) and then incubated for 15 min at 0°C. The mixtures were centrifuged at 13 000 *g* for 10 min at 0°C. Nuclei pellet was resuspended in 30 μl of extraction buffer (20 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 0.5 mM (DTT), 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride

(PMSF)) and mixed briefly on a vortex. After standing for 15 min at 0°C, the mixture was centrifuged for 10 min at 4°C and the supernatant was transferred to a fresh tube. The extracts were diluted with 70 μl of 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.5 mM DTT, 20% glycerol, 0.2 mM EDTA, and 0.5 mM PMSF. Protein concentration was determined by using a BCA Protein Assay Kit (Sigma).

The gel retardation assay was conducted through the methods of Adcock *et al*. (1995) and Guo *et al*. (1995). The double-stranded oligonucleotides for the AP-1 (Promega) were 5′-end labeled with [α-^{32}P]ATP using T4 polyncleotide kinase and purified by ethanol precipitation. Binding reactions were carried out in the buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 0.05% NP40, 2 μg of poly (dI-dC). Nuclear extracts of HT1080 cells were incubated with radiolabeled oligonucleotide ($\sim$5 fmol, 10 000 c.p.m.) for 20 min at 25°C.

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