Resveratrol inhibits matrix metalloproteinase-9 transcription in U937 cells[1]

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KEY WORDS resveratrol; matrix metalloproteinases; phorbol esters; U937 cells; transcription factor AP-1

ABSTRACT

AIM: To examine the inhibitory effect of resveratrol on matrix metalloroteinase-9 (MMP-9) and explore its mechanism. **METHODS:** MMP-9 activity was analyzed by gelatin zymography; MMP-9 protein was detected by Western blot; MMP-9 mRNA expression was investigated by RT-PCR. Activation of activator protein -1 (AP-1) was measured by electrophoretic mobility shift assay (EMSA). **RESULTS:** MMP-9 activity in U937 cells increased significantly after exposed to PMA at 10 nmol/L for 24 h without FCS ($P<0.01$). Resveratrol at 1 and 10 μmol/L showed significant inhibition on MMP-9 activity ($P<0.05$ and $P<0.01$, respectively). Western blot and RT-PCR experiments displayed that MMP-9 protein ($P<0.01$) and mRNA expression ($P<0.01$) increased significantly in PMA-treated U937 cells. Resveratrol at 1 and 10 μmol/L showed inhibitory effects on MMP-9 protein production and MMP-9 mRNA expression ($P<0.05$). The activation of AP-1 induced by PMA was also extensively inhibited by resveratrol at 0.1, 1, and 10 μmol/L. **CONCLUSION:** The inhibitory effect of resveratrol on MMP-9 activity may be partly through suppression of activation of nuclear transcription factor AP-1, and inhibition of MMP-9 mRNA expression and MMP-9 protein production.

INTRODUCTION

Remodeling of the extracellular matrix during normal development and in response to tissue injury and inflammation is thought to be accomplished, in part, by the properly regulated production of matrix metalloproteinases (MMPs)[1]. As a family, these enzymes can degrade essentially all extracellular matrix components, and hence, they have been implicated in normal remodeling processes, such as uterine involution, angiogenesis and wound healing. However, inappropriate expression of these proteinases is thought to contribute to the pathogenesis of various conditions, such as arthritis, periodontal disease, atherosclerosis and cancer[2]. Notably, MMP-9 has been localized to resident and infiltrated inflammatory cells.

Resveratrol (*trans*-3,4',5-trihydroxystilbene), is a natural phytoalexin found in grapes and other plants that has anti-cancer and anti-inflammatory effects. In addition, resveratrol was found to have antioxidant properties and chemopreventive activity. Resveratrol also suppresses the expression of inducible nitric oxide synthase and cyclooxygenase-2, which is likely to contribute to both its anti-inflammatory and anti-oncogenic mechanism[3].

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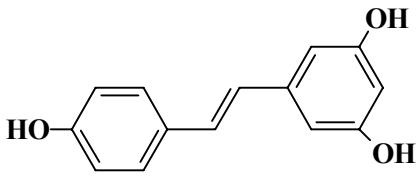

Structure of resveratrol

Human mononuclear phagocytes at various stages of differentiation provide an appropriate system to study the mechanisms regulating inflammatory protease production. For current studies, U937 cell was used. U937 cell line is a human promonocytic cell line, which under basal conditions contains predominantly neutrophil elastase and cathepsin G similar to immature monocytes. When U937 cells are stimulated by phorbol 12-myristate 13-acetate (PMA), expression of metalloproteinases, particularly MMP-9 is induced and enhanced greatly[4]. This process mimics the differentiation of monocytes under inflammatory circumstance in vivo. In the present study, the effects of resveratrol on MMP-9 activity and gene expression in U937 cells were investigated to explore the relationship between anti-inflammatory actions and inhibition of MMP-9.

MATERIALS AND METHODS

Reagents RPMI-1640 medium, TRIzol reagent and M-MLV reverse transcriptase were from GIBCO-BRL. Taq DNA polymerase was from TaKaRa and T4 polynucleotide kinase was from Pharmacia. PMA and AP-1 probe were from Promega. Dexamethasone was from Sigma. Goat monoclonal anti-human MMP-9 antibody was from R&D Systems, Inc. Rabbit anti-goat IgG-AP was from Santa Cruz Biotechnology, Inc. NBT/BCIP Western blot detection reagent was from SABC. Resveratrol was a kind gift from Professor LING Mao, Institute of Materia Medica, Chinese Academy of Medical Sciences.

Cell culture and experimental treatment Human monocytic cells (U937) were obtained from Cell Center, Chinese Academy of Medical Sciences & Peking Union Medical College. Cells were maintained in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS), penicillin 100 kU/L and streptomycin 0.1 g/L. Cells were seeded at a density of 2×10^9 cells/L and cultured at 37 ºC in 5% CO_2. Cells were preincubated for 60 min with resveratrol or vehicle (0.2 % Me_2SO) and treated with PMA without FCS. After an incubation of 24 h, the culture supernatants were gathered for subsequent analysis.

Gelatin zymography Zymography was used for semi-quantitative analysis of gelatinase[5]. The cells supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % polyacrylamide containing 0.1 % SDS and 1 g/L gelatin under nonreducing conditions without prior boiling. After electrophoresis, gels were washed in 2.5 % Triton X-100 for 1 h to remove SDS and allow protein to renature, and subsequently immersed in a mixture containing Tris-HCl 50 mmol/L (pH 7.5) and $CaCl_2$ 20 mmol/L for 16 h at 37 ºC. The gels were then stained with 0.25 % Coomassie Brilliant Blue R250/40 % ethanol/10 % acetic acid, and destained in 25 % ethanol/8 % acetic acid. Enzymatic activities were detected as clear bands of gelatin lysis against a blue background. To measure the relative gelatinase levels, clear zones of gelatinase were scanned with Kodark systems.

Western blot analysis The supernatants of U937 cells were separated by 7.5 % SDS-PAGE, then electrotransfered to a Nitrocellulose membrane. The MMP-9 protein was confirmed by goat anti-human MMP-9 monoclonal antibody (1:1000 dilution) as the primary antibody and rabbit anti-goat IgG-AP (1:2000 dilution) as the secondary antibody. The blots were probed with NBT/BCIP Western blot detection system.

RT-PCR analysis Total RNA was isolated from U937 cells with TRIzol according to the manufacturer's instruction. First strand cDNA synthesis was performed using random hexamers. The sequence of primers are as follows: MMP-9 sense 5'-CGG GAC GGC AAT GCT GAT- 3'; antisense 5'-AGG GCG AGG ACC ATA GAG G-3'; GAPDH sense 5'- GAG GGG CCA TCC ACA GTC TTC -3'; antisense 5'-CAT CAC CAT CTT CCA GGA GCG -3'. MMP-9 PCR reactions were denatured at 95 ºC for 5 min followed by 28 cycles at 95 ºC for 1 min, 57 ºC for 1 min, and 72 ºC for 1 min, and then an extension step cycle at 72 ºC for 10 min. GAPDH PCR reactions were denatured at 94 ºC for 5 minutes followed by 28 cycles at 94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min, and then an extension step cycle at 72 ºC for 10 min. The final products were detected with 2 % agarose gel in TBE buffer in the presence of ethidium bromide, and the bands were visualized under UV light and photographed.

Nuclear extract preparation and EMSA The experiments were performed as described previously[6].

U937 cells were washed twice with D-Hanks' balanced salt solution, resuspended in Buffer A (HEPES 10 mmol/L, $MgCl_2$ 1.5 mmol/L, KCl 10 mmol/L, DTT 0.5 mmol/L, Triton X-100 0.05 %) and then disrupted in Pestle B. After centrifugation, nuclei were resuspended in Buffer C (HEPES 20 mmol/L, glycerol 25 %, NaCl 420 mmol/L, $MgCl_2$ 1.5 mmol/L, EDTA 0.2 mmol/L, PMSF 0.5 mmol/L, DTT 0.5 mmol/L) and lysed on ice. Supernatants were collected, diluted with Buffer W (HEPES 20 mmol/L, KCl 20 mmol/L, $MgCl_2$ 1 mmol/L, DTT 2 mmol/L, PMSF 1 mmol/L, glycerol 17 %). Oligo-nucleotides used for the gel shift analysis were as follows: AP-1 (5'- CGC TTG ATG AGT CAG CCG GAA -3,5'- TTC CGG CTG ACT CAT CAA GCG-3'), mut-AP-1 (5'- CGC TTC ATC AGT CAG CCG GAA-3',5'- TTC CGG CTG ACT GAT GAA GCG-3'). Nuclear extracts were incubated with ^{32}P-labeled oligo-nucleotides, solved on non-denaturing 8 % polyacrylamide gel and visualized by autoradiograph.

Statistical analysis Data were expressed as mean±SD. Comparisons between 2 groups were made by unpaired *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

PMA induced MMP-9 activity in U937 cells MMP-9 activity was very low in unstimulated U937 cells, but with exposure to PMA, MMP-9 activity increased markedly. The data in Fig 1 showed that when PMA was administered at concentrations of 10 pmol/L, 0.1 pmol/L, 1 nmol/L, 10 nmol/L, and 0.1 μmol/L to U937 cells, much higher activity of MMP-9 was observed. The increase of MMP-9 activity by 10 % FCS was also observed in U937 cells.

Resveratrol and dexamethasone inhibited MMP-9 activity induced by PMA The data in Fig 2 showed that resveratrol and dexamethasone suppressed MMP-9 activity in U937 cells exposed to PMA. The inhibitory effect of resveratrol increased as concentration increased. Resveratrol at 10 μmol/L showed potential inhibitory effect, which was almost as strong as that of dexamethasone at 10 μmol/L.

PMA 10 nmol/L alone inhibited U937 cell proliferation, while dexamethasone or resveratrol alone showed no toxicity to U937 cells. Dexamethasone or resveratrol combined with PMA 10 nmol/L showed no additional toxicity to U937 cells *vs* PMA group (data not shown). The above results indicated that cytotoxicity did not contribute to the inhibition of MMP-9 activity.

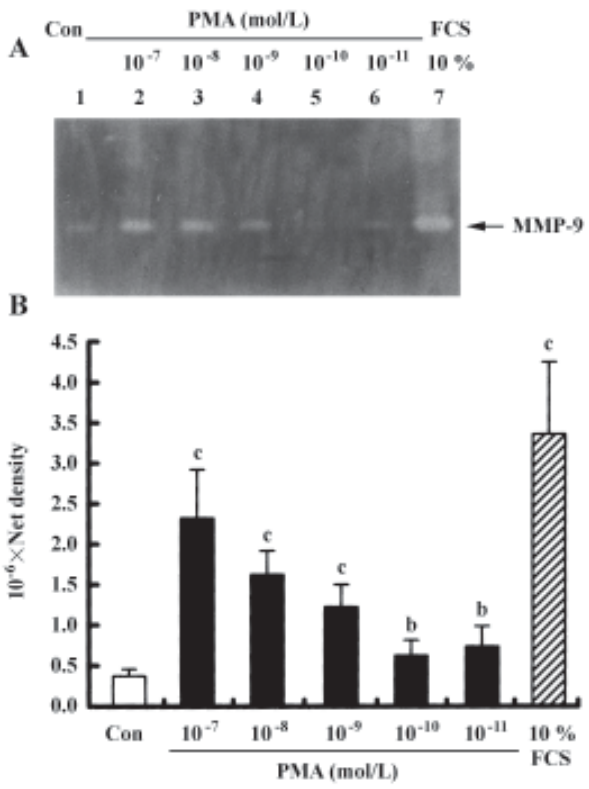

Fig 1. Induction of PMA and FCS on MMP-9 activity of U937 cells. (A) U937 cells were exposed to different concentrations of PMA or 10 % FCS for 24 h, the activity of MMP-9 in supernatants was analyzed by gelatin zymography. Lane 1: Control; Lane 2-6: PMA 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, 1×10^{-10}, 1×10^{-11} mol/L, respectively; Lane 7: 10 % FCS. (B) To determine the relative MMP-9 levels, MMP-9 bands detected as clear zones of gelatin lysis were scanned with Kodak system. The MMP-9 levels were represented by net densities. *n*=4. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

Resveratrol and dexamethasone inhibited MMP-9 protein production induced by PMA To determine whether the above inhibition (data shown in zymography result) is related to direct inhibition of MMP-9 enzyme activity or related to inhibition of MMP-9 protein expression, Western blot assay of MMP-9 protein in cultured supernatants was carried out. Fig 3 showed that MMP-9 protein was induced by PMA10 nmol/L. Co-treatment with resveratrol or dexamethasone caused a decrease in PMA-mediated MMP-9 protein production. The maximal drug effect was observed at 10 μmol/L.

Resveratrol and dexamethasone inhibited the increase of MMP-9 mRNA induced by PMA To further elucidate the mechanism responsible for the changes in amounts of MMP-9 protein, the steady-state levels

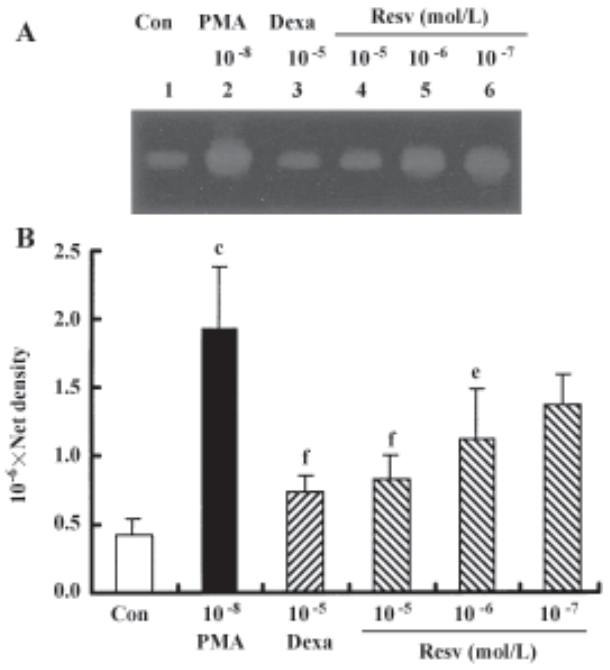

Fig 2. Inhibition of dexamethasone (Dexa) and resveratrol (Resv) on activity of MMP-9 secreted from U937 cells exposed to PMA for 24 h. (A) Lane 1: Control; Lane 2: PMA 1×10^{-8} mol/L; Lane 3: dexamethasone 1×10^{-5} mol/L; Lane 4-6: resveratrol 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} mol/L, respectively. (B) To determine the relative MMP-9 levels, MMP-9 bands detected as clear zones of gelatin lysis were scanned with Kodak system. The MMP-9 levels were represented by net densities. n=4. Mean±SD. $^{c}P<0.01$ *vs* control. $^{e}P<0.01$, $^{f}P<0.05$ *vs* PMA.

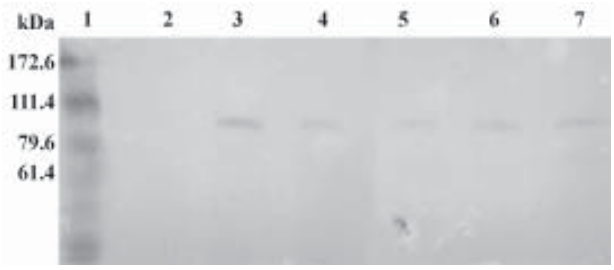

Fig 3. Inhibition of dexamethasone and resveratrol on MMP-9 protein secreted from U937 cells exposed to PMA for 24 h. Lane 1: Prestained protein marker; Lane 2: Control; Lane 3: PMA 1×10^{-8} mol/L; Lane 4: dexamethasone 1×10^{-5} mol/L; Lane 5-7: resveratrol 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} mol/L, respectively. Similar results were obtained in three experiments.

of MMP-9 mRNA was determined. Treatment with PMA resulted in a marked increase in MMP-9 mRNA, an effect that was suppressed by dexamethasone and resveratrol at 10 μmol/L in a concentration-dependent manner (Fig 4).

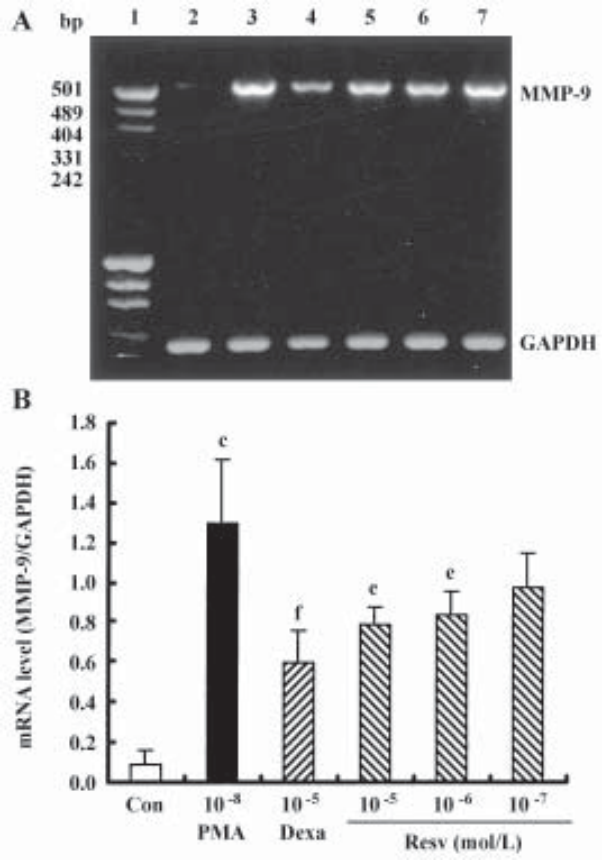

Fig 4. Inhibition of dexamethasone (Dexa) and resveratrol (Resv) on expression of MMP-9 mRNA in U937 cells exposed to PMA for 24 h. (A) Lane 1: PUC19DNA/MspI DNA ladder; Lane 2: Control; Lane 3: PMA 1×10^{-8} mol/L; Lane 4: dexamethasone 1×10^{-5} mol/L; Lane 5-7: resveratrol 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} mol/L, respetively. (B) The mRNA levels were represented by the ratio of net density of MMP-9 and GAPDH. n=4. Mean±SD. $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* PMA.

Resveratrol and dexamethasone inhibited AP-1 activation induced by PMA To determine whether PMA induced activation of transcriptional factors, EMSA assay was also performed using consensus oligonucleotides for AP-1. As shown in Fig 5, PMA induced a substantial increase in AP-1 activation, which could be inhibited by dexamethasone at 10 μmol/L and resveratrol at 0.1, 1, and 10 μmol/L.

DISCUSSION

Considerable evidence has accumulated to suggest that inhibitors of MMP-9 are useful for treating inflammation and preventing cancer. Drugs that interfere with the signaling mechanisms that up-regulate MMP-9 should also be useful in this regard because they both

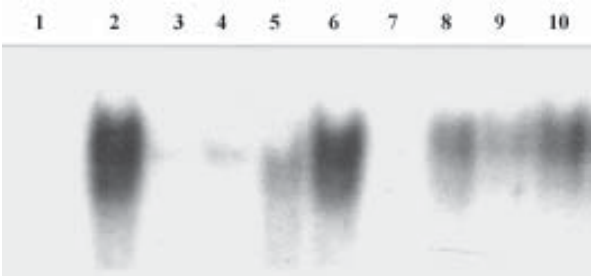

Fig 5. Inhibition of dexamethasone and resveratrol on activation of AP-1 in U937 cells exposed to PMA for 24 h. Lane 1: Probe alone; Lane 2: AP-1; Lane 3: AP-1+competitive none ^{32}P-labeled probe; Lane 4: AP-1 mut probe; Lane 5: control; Lane 6: PMA 1×10^{-8} mol/L; Lane 7: dexamathasone 1×10^{-5} mol/L; Lane 8: resveratrol 1×10^{-5} mol/L; Lane 9: resveratrol 1×10^{-6} mol/L; Lane 10: resveratrol 1×10^{-7} mol/L. Similar results were obtained in three experiments. Nuclear extracts were prepared from cultured U937 cells treated with PMA and Dexa or resveratrol indicated above and incubated with ^{32}P-labeled oligonucleotides encompassing AP-1 or mutational (mut) consensus motifs followed by analysis with EMSA. In lane 3 a 100-fold molar excess of unlabeled specific oligonucleotide was added to the binding reactions.

decrease total MMP-9 activity. It showed in the present experiments that resveratrol suppressed PMA-mediated induction of MMP-9 activity by inhibiting MMP-9 gene transcription. Resveratrol was found recently to have little direct inhibitory effect on MMP-9 enzyme activity[7], so inhibition of resveratrol on MMP-9 gene expression may be one of its anti-inflammatory mechanisms.

In regard to the mechanism by which resveratrol modulates gene expression, resveratrol was found to suppress PMA-mediated activation of AP-1 in U937 cell. AP-1 has been implicated in promoting carcinogenesis[8]. This effect is likely to contribute to the anti-tumor activity of resveratrol.

Resveratrol has been reported to exhibit a wide range of pharmacological properties not only *in vitro* but also *in vivo*. The anti-inflammatory property of resveratrol was demonstrated by suppression of carrageenan-induced paw edema, an effect attributed to suppression of PG synthesis via direct, selective inhibition of COX-1[8]. In our previous work, resveratrol was shown to inhibit mouse ear edema induced with croton oil when administrated subcutaneously. This effect contributed partly to suppression of MMP-9 expression. As to the delayed-type hypersensitivity induced with DNFB, resveratrol also showed certain inhibitory effect. The common problem exposed in these experiments is that: although resveratrol has been demonstrated to have such extensively pharmacological properties, the effective dose of resveratrol is very high. It suggested that resveratrol should be considered not only as a common constituent of the human diet only, but also as a more powerful and selective drug based on the further investigation of the relationship between its structure and different functions.

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