

Suppression of Thrombospondin-1 Expression by PMA in the Porcine Aortic Endothelial Cells

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Received November 4, 2003 / Accepted December 20, 2003

Thrombospondin-1 (TSP-1), a negative regulator in tumor growth and angiogenesis, is cell-type specifically regulated and at transcriptional level by external stimuli. Previously, we found that phorbol 12-myristate 13-acetate (PMA) suppressed TSP-1 expression in porcine aortic endothelial (PAE) cell, but enhanced in hepatoma cell line, Hep 3B cell. A region between -767 and -723 on the *tsp-1* promoter was defined as a responsive site to the suppression in PAE cell. Based on the previous results, the molecular mechanism of TSP-1 expression was determined by characterizing interactions between *cis*-elements and *trans*-factors using three overlapped oligonucleotide probes, oligo a-1 (from -767 to -738), a-2 (-759 to -730) and a-3 (-752 to -723). The results from electromobility shift assay showed that PMA-induced suppression of TSP-1 transcription in PAE cell might be caused via a negative regulator binding to the region from -752 to -730 and additionally generated by lacking two positive regulators binding to the sites from -767 to -760 and from -752 to -730. Especially, PMA enhanced the binding ability of the negative regulator to the site from -752 to -730 in PAE cell, but anti-c-Jun did not affected its binding ability.

Key words – Thrombospondin-1, c-Jun, gene expression, promoter activity, *cis*-elements, *trans*-acting elements.

It has been extensively reviewed that thrombospondin-1 (TSP-1) is ubiquitously synthesized in human and animal cells and implicated in a number of processes pertaining to various cellular functions [3,5,13]. Interestingly, increasing evidences indicate a role of TSP-1 as a negative regulator of solid tumor progression and angiogenesis [4,11,19,23,24].

Angiogenesis is due to an imbalance between positive and negative factors, such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), angiostatin, endostatin and TSP-1, among others. In comparison between TSP-1 and VEGF in mRNA level (ratio of TSP-1 mRNA/VEGF mRNA), high ratio was closely associated with poor vascularity [12]. Therefore, it is clinically important to investigate the detailed mechanism of TSP-1 expression for maintaining its optimal level. In addition, if human-specific promoter could be used in the pig cell with preserved activity and specificity, there would be no need to isolate and characterize the porcine counterparts.

It is well known that many genes are cell-type specifically expressed, depending on interactions between *cis*-acting elements and *trans*-acting factors in response to extracellular stimuli including growth factors, cytokines

and chemical agents. Particularly, an initial response of cells to phorbol 12-myristate 13-acetate (PMA) involves the induction of several genes known as “transcription regulating genes” including Sp-1, early growth response-1 (Egr-1) and c-Jun [1,7,13,18]. Previously, we measured TSP-1 promoter activities and identified the responsive site to PMA-induced suppression, which was located at a region from -767 to -723 on its promoter [13]. This responsive site contains putative STRE, Egr-1, GATA-1 and Sp-1 binding sites [3]. It has been known that Sp-1 binds to one or more GC boxes located near a transcriptional start site [8,9]. Additionally, its novel role was recently reported that it bound to the C-rich element far from the transcriptional start site [7]. Since the consensus sequences of Egr-1 and Sp-1 binding site resembles each others, Egr-1 reduces mouse thrombospondin-1 transcription by competing with Sp-1 [20]. Recently, a significant evidence supports that Egr-1 can function as coactivators of c-Jun by forming Egr-1/c-Jun complex and subsequently regulate the target gene expression controlled by c-Jun [16]. In our report, we have previously demonstrated that PMA upregulated TSP-1 expression in hepatoma Hep 3B cells resulting from increased c-Jun binding to the AP-1 site (-1492) on the *tsp-1* promoter [14]. But PMA downregulated TSP-1 expression in human monocytes [25] and rat fibroblast [8,17] involving

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increased c-Jun. We also reported that TSP-1 expression was lowered by PMA in porcine aortic endothelial (PAE) cell [13].

In this study, based on the previous results, we examined the molecular mechanism of TSP-1 gene expression through interactions between *cis*-acting element and *trans*-acting factor using PMA-treated PAE cells. Now, we obtained two noticeable results from electrophoretic mobility shift assay (EMSA) that PMA-promoted suppression of TSP-1 transcription in PAE cell might be mainly caused via a negative regulator binding to a site between -767 and -723. Moreover, this reducing effect might be mediated by lacking two unknown positive regulators binding to the region from -767 to -723. As inferring from commonly overlapped sequences of the bound probes, these positive regulators bound to the site from -767 to -760 and from -752 to -730, respectively. Binding potentiality of the site from -767 to -760 was affected by specific antibody to c-Jun in Hep 3B cells but not by any specific antisera to Sp-1, Egr-1 and GATA-1, their consensus sequences of which are detected in this region.

Taken together, these data suggest that PMA-induced suppression of TSP-1 expression be additively provoked by a negative regulator binding in addition to the loss of two positive regulators binding to the target motives on the *tsp-1* promoter in PAE cell.

Materials and Methods

Cells and culture conditions

Hepatoma cell line Hep 3B cells were cultured in Dulbecco's modified Eagle's medium and Porcine aortic endothelial cells (PAE) (passage 12~20) in M199 medium, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), respectively, and were maintained in a humidified incubator at 37°C with 5% CO₂.

Western blot analysis

Cells were initially seeded on 35-mm dishes at a density of 1×10^5 cells/ml. After culture in the presence of 10% serum for 24 h, and cells were serum-free starved for 16 h and then treated for various time periods with PMA at various concentrations. Conditioned media were collected and concentrated as written in previous report [14]. Protein concentrations were quantified by the bicinchoninic acid method using BCA (Bicinchoninic Acid) protein assay

reagent (Pierce, Rockford, IL). 20 µg of protein from each supernatant was separated on 8% SDS-PAGE. After electrophoretic transfer of the protein from the polyacrylamide gel to nitrocellulose membrane (Schleicher and Shuell, Dassel, Germany), the membrane was blocked by incubating with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) overnight at 4°C. The membrane was incubated with monoclonal antibody against mouse TSP-1 antibody diluted 1:1000 in TBS containing 3% nonfat dried milk for 1 h at room temperature, then washed three times with TBS-T and incubated for 1 h at room temperature with 1:1000 dilution of peroxidase-conjugated anti-mouse immunoglobulin antibody (sigma) in TBS containing 3% dried nonfat milk. The membrane was washed three times with TBS-T and immunocomplex was visualized by enhanced chemiluminescence using the ECL kit (Amersham-pharmacia, UK).

Northern blot analysis

Total RNA from the semiconfluent cells on 35-mm dishes was extracted using STAT-60 (TEL-TEST Inc.). RNA samples (10 µg/lane) were separated on agarose gel containing 6% formaldehyde, transferred onto nylon membrane (Schleicher and shuell), and UV cross-linked with a Stratalinker UV light source (Stratagene, La Holla, CA). TSP-1 probe was labeled with DIG-11-dUTP (Boehringer Mannheim). The membrane were prehybridized for 1 h at 55°C in a buffer containing 50% formamide, 0.75 M NaCl, 0.075 M trisodium citrate pH 7.0, 5% blocking reagent (Amersham Life Science, USA). The membrane was hybridized in the same buffer overnight at 55°C with DIG-11-dUTP-labelled TSP-1 cDNA probe. The membrane were then washed twice in $2 \times$ SSC containing 0.1% SDS at room temperature and finally twice in 0.1% SSC containing 0.1% SDS at 60°C. The membrane was developed by immunochemical method using DIG chemiluminescent detection kit (Boehringer Mannheim, Germany). Human GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA was used as internal control for the loading of RNA.

Reporter constructs

The *tsp-1* promoter fragment was amplified by PCR with rTth DNA polymerase, XL (PE Applied Biosystems, Foster city, CA) from genomic DNA of Hep 3B cells using the following primers: Identical reverse primer was used for each construct, which had the sequence pTSP-LUC+754,

5'-GATCCTGTAGCAGGAAGCACAAAG-3' ; Their forward primers were pTSP-LUC-2200, 5'-GAGGAAGGGCTTTGT-GTTTGAGATA-3', pTSP-LUC-1182 5'-GAGATGAATGGA-ATTCCAGGAAGCT-3', pTSP-LUC- 860 5'-GTTTTCAAG-ACACAATTTCAATTT-3', pTSP-LUC-767 5'-CCCCT TGA-TGAGAATACGCACACCG-3', pTSP-LUC-655 5'-AAGGC-AGCGAAGGC GAACGCGGCGC-3', pTSP-LUC-521 5'-TG-GAGGAGAGTCAGCGAGGGCCCG A-3', pTSP-LUC-407 5'-TATCCACCTCTCGCCATCAACCAGG-3', pTSP- LUC-45 5'-GGAATGCGAGCGCCCTTTAAAAGC-3', These forward primers were ligated to a *Kpn* I- linker at the 5' end, and the reverse primer had a *Xho* I-linker at the 5' end. All fragments were gradually shortened by serial deletion from the 5' end, but have the identical 3' end (+754 region). PCR was performed for 30 cycles : 94°C for 5 min, 94°C for 30 sec, 50°C for 30 sec, 72°C for 3 min, followed by a final extension step at 72°C for 5 min. Amplified fragments were digested with *Kpn* I and *Xho* I. The resulting fragments were then introduced in front of a luciferase gene of the pGL3-basic vector (Promega, Madison, WI).

Transient transfection and reporter assay

Hep 3B cells were seeded on 12-well plates at 1×10^5 cells/ml and incubated for 24 h. Prior to transfection, the cells were cultured in 0.4 ml of OPTI-MEM (Gibco/BRL) for 30 min. 0.5 μ g each of serial deletion constructs of TSP-1 promoter inserted into pGL-basic vector were incubated with 100 μ l OPTI-MEM containing 2 μ l Plus reagent (Invitrogen) for 15 min at room temperature and then 100 μ l Opti-MEM containig 2 μ l LipofectAMINE (Invitrogen) was added. The mixture was incubated at room temperature for an additional 30 min and added to the culture media, and incubated at 37°C for 2 h. One milliliter of DMEM containing 10% FBS was then added to each well and the cells were incubated overnight at 37°C. The cells were treated with 50 nM PMA and incubated for 24 h before harvest. Cell lysates were prepared by extraction with 100 μ l of reporter lysis buffer (Promega). Luciferase (LUC) activity was measured using a TD-20/20 Luminometer (Turner Designs). The β -galactosidase activity was used as an internal control to normalize for transfection efficiency. Relative luciferase activity was expressed as mean \pm S.D. in two or three separate experiments.

Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared as follows : Cells (1

$\times 10^6$) were harvested with rub scraper, suspended with gentle pipetting in 200 μ l of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.2 mM Na_3VO_4 , 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 0.6% Nonidet P-40) and incubated on ice for 15 min. After centrifugation at $12,000 \times g$ for 30 sec, the supernatants were saved as a cytoplasmic extracts. The pellets were washed once with the same volume of buffer A, resuspended in a 100 μ l of extraction buffer B (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4 , 1 μ g/ml leupeptin, 1 μ g/ml aprotinin) and incubated on ice for 1 h. After centrifugation at $12,000 \times g$ for 30 min, the supernatants were harvested as the nuclear protein extracts. The protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) and stored at -70°C until use. The following 45-bp oligonucleotides and their complements were synthesized : 5'-CCCCTTGATGAGAGAATACGCACACCGCCCCCAAGCGGCCGGCCGAG-3' (Oligo a, nucleotides (nts) -767 to -723 on the TSP-1 promoter), 5'-CCCCTTGATGAGAGAA-TACGCACACCGC CCCC-3' (Oligo a-1, -767 to -738), 5'-AGAGAATACGCACACCGCCCCCAAG CGGCC-3', (Oligo a-2, -759 to -730) 5'-ACGCACACCGCCCCCAAGCGGCCG GCCGAG-3' (Oligo a-3, -752 to -723). Annealed Double-stranded oligonucleotides were [^{32}P]-end-labeled using T4 polynucleotide kinase. Binding reactions were carried out for 20 min at room temperature using 5 μ g of nuclear protein in a final volume of 20 μ l reaction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 0.5 μ g poly (dI-dC), 4% glycerol, and 5×10^5 cpm of [^{32}P]-labeled oligonucleotide probe. The protein-DNA complexes were separated on 6% native polyacrylamide gels using 0.5 \times Tris borate-EDTA buffer (TBE) at 170 V for 2 h, dried and autoradiographed at -70°C overnight. For supershift analysis, 1 μ g of a specific antibody was preincubated for 30 min at 4°C with the nuclear extract prior to the addition of the radiolabeled probe.

Results

Effects of PMA on TSP-1 expression

To characterize mRNA and protein levels of the PMA-stimulated TSP-1 expression, the serum-starved PAE cells were incubated with PMA under various conditions depending on time or concentration. As shown in Fig. 1A,

its mRNA level began to decrease after 6 h and slowly decreased thereafter, but undetectable 24 h later (data not shown). According to the results from Western blot analysis depending on time- course (Fig. 1B), TSP-1 level in the culture supernatant constantly appeared until 6 h and was sharply reduced thereafter. In additional experiment, to determine the response of PAE cells to different dose of PMA, cells were stimulated for 20 h with PMA at the concentrations, 10, 25, 50, 75 and 100 nM, and TSP-1 level in the harvested culture supernatants or cell lysates was evaluated (Fig. 2). Notice ably, secreted TSP-1 level was consistently kept in the presence of PMA, while cytosolic TSP-1 was remarkably diminished at even low dose, 10 nM (Fig. 2A). Consequently, to determine the change patterns of the total TSP-1 value, 10 µg protein in the culture supernatant was mixed with 10 µg protein in the cell lysate and performed by Western blot analysis. Figure 2B clearly showed that PMA down-regulated total TSP-1 in dose-dependent manner. Since the secreted TSP-1 level was constantly maintained by PMA at various dose, we confirm the possibility whether its constant level was generated by dose-dependent secretion without new production. Both

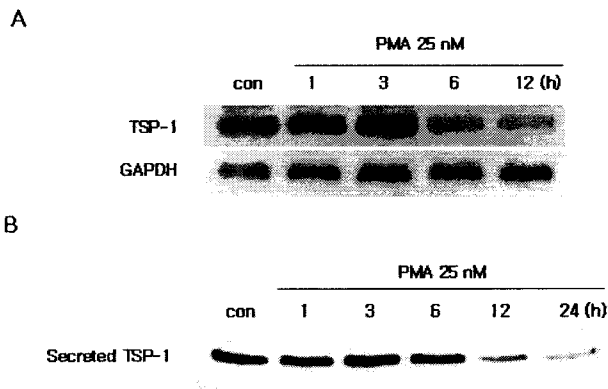


Fig. 1. Time-dependent suppression of TSP-1 in the PMA-treated PAE cell. 2×10^7 cells were cultured for 48 h in the media containing 10% serum and incubated with 25 nM PMA for the indicated time periods under serum-starved condition. To determine the TSP-1 mRNA level, 2 µg of total cellular RNA was carried out Northern blot and hybridized with human TSP-1 cDNA probe. The same membrane was rehybridized with the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. To estimate the TSP-1 protein level culture supernatants were analyzed by Western blotting using polyclonal anti-TSP-1 antibody. The migrated bands were visualized by enhanced chemiluminiscent (ECL) detection system.

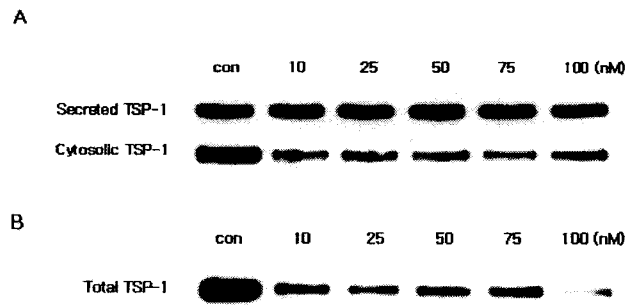


Fig. 2. Dose-dependent TSP-1 protein level existed as secreted or cytosolic (A) and total TSP-1 (B) in the PAE cells. Cells were treated with various dose of PMA from 10 to 100 nM. Concentrated culture supernatant and cell lysate were Western blotted, respectively. 10 µg protein from the culture supernatant was mixed with 10 µg protein from the cell lysate to prepare the total TSP-1 and the mixture was also Western blotted.

secreted and cytosolic TSP-1 levels were estimated at various time periods (Fig. 3). In the unstimulated PAE cells, cytosolic TSP-1 independently existed to time periods from 1 to 48 h, but the secreted TSP-1 gradually increased (Fig. 3A). The data obtained from the unstimulated cells indicated that TSP-1 was constantly synthesized and continuously secreted into extracellular medium in the absence of PMA. Oppositely, PMA-influenced reduction was remarkable in the level of secreted form, which was detected at constant level 3 h later and then dramatically declined after 6 h, whereas the cytosolic level slightly

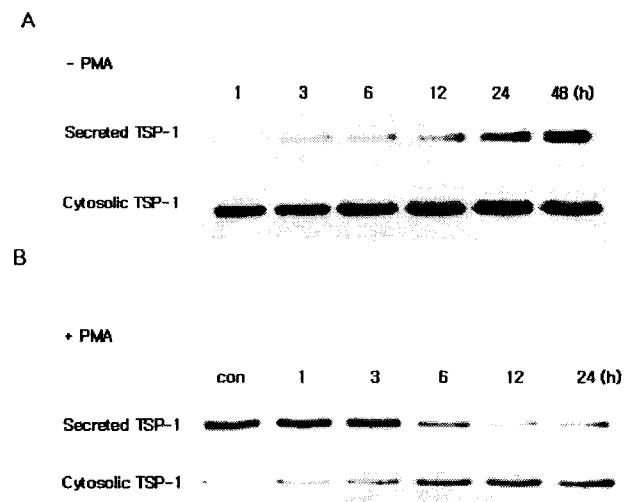


Fig. 3. Time-dependent changes of the secreted or the cytosolic TSP-1 level derived from the unstimulated (A) and the PMA-stimulated (B) PAE cells. Cells were treated with 50 nM PMA during various time periods as indicated.

elevated after 6 h and this level was kept for 24 h (Fig. 3B). In coupled with the change patterns of both cytosolic and secreted levels, the elevated level of cytosolic form depending on time resulted from retarding the secretion of pre-existed TSP-1 in the cytosol without new synthesis.

Identification of the responsive sites to PMA-induced down regulation of TSP-1 expression

Because PAE cell, a primary normal cell, was unstable and its cellular functions could be often altered depending on culture passage, we reevaluated the *tsp-1* promoter activity expressed by eight 5'-serially truncated constructs to reconfirm the results from the previous report as shown in Fig. 4A [13]. As expected, four constructs bearing the region up to -655 strongly decreased their transcriptional abilities in the presence of PMA, but further deletion, up to -45, increased the transcriptional activity in the response to PMA (Fig. 4B). Thus, the region from -767 to -655 contains the *cis*-element responsive to the PMA-dependent

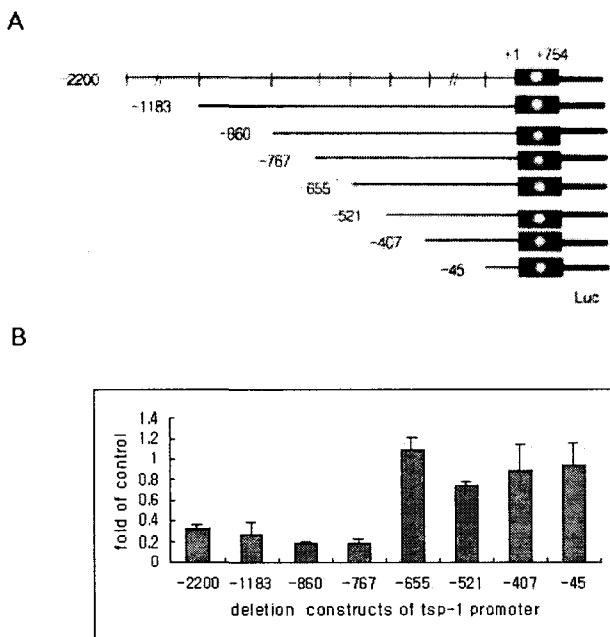


Fig. 4. Schematic structure of eight 5' serial deletion constructs and their transcriptional activities.

PAE cells were transiently transfected with each 5' deletion construct and treated with 100 nM PMA for 12 h. Transcription activity was determined by measuring luciferase activity yielded from each reporter plasmid and expressed as relative activity to the luciferase activity of the unstimulated control cells. The specific luciferase activity was normalized to the cotransfected β -gal activity. The data represent the mean \pm S.D. of the three separate experiments.

repression of TSP-1 expression. Previously, we have defined the responsive site located at a region from -767 to -723 on the human *tsp-1* promoter [13] by narrowing down the range of target site using the oligonucleotide probe, oligo a. Based on the previous data, to identify the exact *cis*-acting element responsible for PMA-induced suppression, we have made additional three probes derived from following sites from -767 to -738, from -759 to -730 and from -752 to -723 and designated as oligo a-1, a-2 and a-3. In Fig. 5A showing gel mobility shift assay utilizing the 45-mer oligonucleotide corresponding to -767~-723 as a probe, an unknown nuclear protein bound to this probe, which was indicated as the solid arrow in Hep 3B cells. Although a supershifted band was not detected, the band intensity was significantly decreased by anti-c-Jun. Moreover, in the third lane of Fig. 5B (tailed star), the supershifted band was clearly detected. To investigate the exact binding site through narrowing down the sequence range in the binding site, nuclear extracts were incubated with labeled three probes, oligo a-1, a-2 and a-3, respectively and each binding affinity was analyzed in Hep3B cell. The band disappearance by anti-c-jun was observed by only oligo a-1, while it was not observed by oligo a-2 and a-3 (closed arrow in Fig. 5B). Thus, the unknown nuclear protein bound to a region between -767 and -738, and its binding activity was affected by anti-c-Jun in Hep 3B cells. Although another nuclear protein was complexed with three probes, oligo a, a-2 and a-3 in Hep 3B cell, their binding affinity were not changed by anti-c-Jun (slanted arrow in Fig. 5B). From the result, the shifted band pointed by slanted arrow in Fig. 5B might result from another nuclear protein binding to the region between -752 and -730 corresponding to the overlapped site between two probes (a-2 and a-3). In PAE cells, a nuclear protein bound to the oligo a was detected and the band intensity was enhanced by treating cells with PMA (Fig. 5C; open arrow). This binding activity in PAE cells was not observed by oligo a-1. Unexpectedly, it was observed by oligo a-2 and a-3, and not affected by anti-c-Jun (Fig. 5C). In coupled with the shifted band generated by oligo a-2 and -3, an unknown nuclear protein in PAE cell bound to the region between -752 and -730 and its binding affinity was increased by PMA, but was not affected by anti-c-Jun.

Since the band positions obtained from oligo a or a-3 in Hep 3B cell were different from those in PAE cell, differences in response to oligo a or oligo a-3 between both

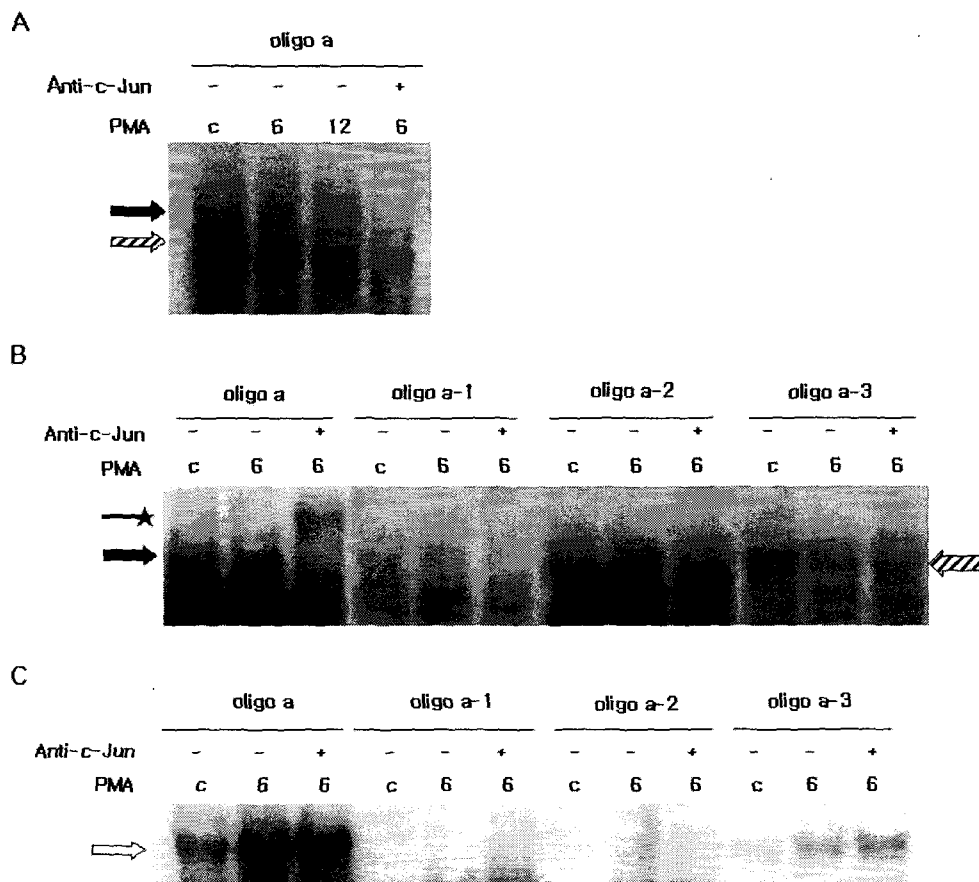


Fig. 5. Gel mobility shift and supershift analysis of nuclear proteins obtained from the PMA-stimulated Hep 3B (A and B) or PAE cells (C).

Four probes were composed of the nucleotide sequences corresponding to regions from -767 to -723 (oligo a), from -767 to -738 (oligo a-1), from -759 to -730 (oligo a-2), and from -752 to -723 (oligo a-3), respectively. Supershift analysis was performed by specific antiserum to c-Jun (1:100). Solid arrow points the band position bound with oligo a-1, which was slightly decreased by PMA and completely disappeared by antibody to c-Jun and slanted arrow indicates another band position bound to three probes, oligo a, a-2 and a-3, which is not diminished by anti-c-Jun in Hep 3B cells. The tailed star represents the supershifted band by anti-c-Jun. The bound position of the nuclear protein from PAE cell is indicated by open arrow, which shows strong binding with two probes such as, oligo a, a-3, but weak binding with a-2. Especially, the band intensity indicated by open arrow was increased by PMA.

cells were examined by band position from EMSAs. As shown in Fig. 6, the band detected in PAE cell (open arrow) migrated slightly faster than that of the band bound to the site from -767 to -723 in Hep 3B cell (closed arrow, Fig. 6A), but it did slower than that of the band bound to the site from -752 to -730 in Hep 3B cell (slanted arrow, Fig. 6B).

Discussion

TSP-1 has been reviewed to be a negative regulator in solid tumor progression and angiogenesis [4,11,23,24]. Transfer of TSP-1 cDNA into a human breast carcinoma cell line suppresses primary tumor proliferation, metastasis, and

angiogenesis [24]. Since angiogenesis results from an imbalance between positive and negative factors, high ratio of TSP-1 mRNA against VEGF mRNA is closely associated with poor vascularity [12]. Thus, it is clinically significant that the TSP-1 level is constantly maintained at optimal level.

TSP-1 level in the body fluids are normally very low, but is cell-type specifically regulated by extracellular stimuli [3,14,25]. For example, PMA upregulated TSP-1 level in rat heart endothelial, porcine thyroid and U937 cells, but downregulated in rat embryo fibroblast and human monocyte cells [2,6,8,17,21,22]. An interesting report has shown that c-Jun played as a primary mediator in the PMA-reduced

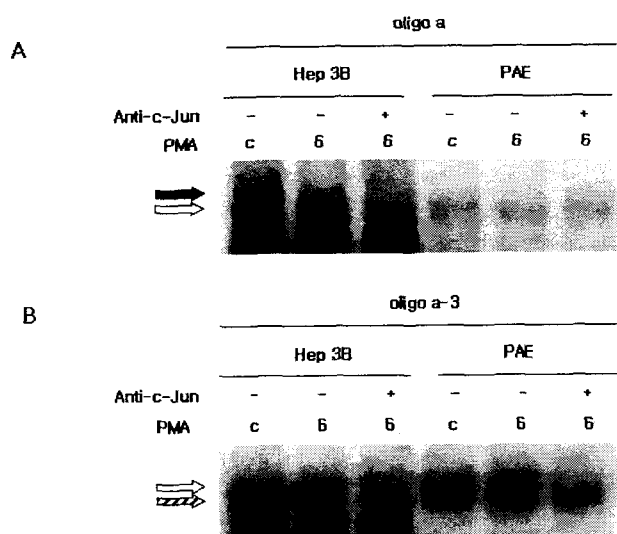


Fig. 6. Gel mobility shift analysis of the oligo a (A) and a-3 (B) with nuclear extracts from Hep 3B and PAE cells to compare the exact position of specifically shifted bands yielded from both cells.

Arrows indicate each specifically shifted band as followings : Solid arrow, the nuclear protein bound to the site between -767 and -723 in Hep 3B cell ; open arrow, the nuclear protein bound to the site between -752 and -730 in PAE cell ; slanted arrow, the nuclear protein bound to the site between -752 and -730 in Hep 3B cell.

TSP-1 expression and WT1 site (nt -280) on the *tsp-1* promoter was defined as the responsive site [8]. Nonetheless, the regulation of TSP-1 expression has been recognized to be of importance and has been studied by many researchers in the last decade [3,4,8,17,20]. Surprisingly, there is relatively little information about the detailed mechanisms of the TSP-1 expression at molecular level.

Recently, our serial reports demonstrated that PMA upregulated TSP-1 expression in Hep 3B cell via increased c-Jun binding to the AP-1 motif (nt -1492) on the *tsp-1* promoter [14] and downregulated in PAE cell [13]. When the cytosolic TSP-1 level was checked in addition to the secreted TSP-1 levels in the unstimulated PAE cell, the cytosolic TSP-1 was retained at constant level until 48 h, but the secreted TSP-1 level gradually increased in time-dependent fashion. Oppositely, in the PMA-treated PAE cell, the secreted level was constant for 3 h and slowly decreased thereafter, while the cytosolic form was slightly elevated for 24 h. These results support that PMA retarded the secretion of TSP-1 from the cytosol without promoting new TSP-1 synthesis and resulted in decreasing the extracellular TSP-1 level.

Especially, we previously reported that a responsive site to the PMA-induced suppression was located at a region between -767 and -723. Based on the previous report, we prepared three oligonucleotide probes, oligo a-1 (-767~-738), a-2 (-759~-730), and a-3 (-752~-723) derived from oligo a corresponding to the site within -767 and -723 to identify the exact responsive site and interacting *trans*-acting factors in response to the PMA-influenced suppression in PAE cell.

In gel shift assay using oligo a, two shifted bands were observed in Hep 3B cell, whereas one band was detected in PAE cell. From two oligo a-bound proteins from Hep 3B cell, one protein interacted with only oligo a-1, while another protein interacted with two probes, oligo a-2 and a-3 (Fig. 5B). Moreover, protein interacted with oligo a-1 was affected by specific antiserum to c-Jun in Fig. 5A. Because of no consensus sequence for c-Jun binding within this region (-767~-723), it supports a possibility that oligo a-1 bound protein interacts with c-Jun and is affected by anti-c-Jun. However, when the band detected with oligo a-3 in PAE cell was compared with those from Hep 3B cell, its migrated position was different (Fig. 6A and B). Furthermore, it also bound with oligo a-2, but its band intensity was very faint (Fig. 5C). Since three bands shifted by oligo a occurred in near position and as trailing patterns in Hep 3B cell, the data obtained by oligo a might misguide to be an identical band. In the previous study [13] using only oligo a, they overlooked the fact that the nuclear protein bound with oligo a-2 and a-3 in PAE cell was different from two proteins derived from Hep3B cell. Coupled with the commonly overlapped sequence of the bound probes, we infer that two nuclear proteins from Hep 3B cell bound to the regions from -767 to -760 (closed arrow) and from -752 to -730 (slanted arrow), respectively, and one nuclear protein from PAE cell bound to the region from -752 to -730. PMA increased the band intensity shifted by oligo a-3 in PAE cell, and its binding affinity was not interfered by anti-c-Jun. These results indicate that c-Jun participates directly in the binding of unknown nuclear protein (closed arrow) to the motif between -767 and -760 in Hep 3B cell and in elevating the unknown nuclear protein expression (open arrow) or its binding affinity to the motif from -752 to -730 in PAE cell. When PMA-induced upregulation in Hep 3B cell is combined with two bands detected in Hep 3B cell lacking in PAE cell, the bound nuclear proteins in Hep 3B cell may be positive

regulators. In considering PMA-mediated reduction in PAE cell, the bound nuclear protein in PAE cell may be a negative regulator.

Hence, it suggest that PMA-dependent reduction in TSP-1 expression be caused by binding of a negative regulator to the site from -752 to -730 in addition to the loss of two positive regulators binding to the site from -767 to -760 and from -752 to -730, respectively. But the mechanism remains uncertain because these regulators bind cell-type specifically to the *tsp-1* promoter. It is not ruled out that two positive regulators binding is prevented by the negative regulator binding in PAE cell. It is widely proved that many *cis*-elements can be controlled by binding of *trans*-acting factors to the target motif. Therefore, we searched putative *cis*-elements in the region from -767 to -723 on the *tsp-1* promoter and found a number of potential regulatory sites including Egr-1, GATA-1, Sp-1 and STRE (stress-response element). A previous report has shown that GATA-1 and Sp-1 were not involved in the PMA-promoted down-regulation of TSP-1 transcription in PAE cell [13]. Likely, according to the gel shift experiments utilizing three overlapping probes, oligo a-1, a-2 and a-3, the overlapped sequences in the commonly bound probes could not contain GATA-1 binding sequence. However, in spite of the existence of Sp-1 consensus sequence at -742 overlapping with Egr-1 motif, any bands were not supershifted by antibody to Sp-1 or Egr-1 (data not shown). Particularly, a putative STRE site is located at -765 which is only contained in the oligo a-1. Since antiserum to STRE factor is not sold commercially, we will performed the supershift analysis after preparation of the antiserum to STRE.

Acknowledgement

This work was supported by Korea Research Foundation (KRF-2002-015-EP00 33).

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초록 : 정상 돼지 대동맥 내피세포에서 PMA에 의한 thrombospondin-1 발현 억제

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암의 성장과 신생혈관 억제인자로 알려진 thrombospondin-1의 생합성은 다양한 외부자극에 대해 전사단계에서 세포 특이적으로 조절된다. 이전의 연구에서 본 연구자들은 PMA가 정상 돼지 대동맥 내피세포(PAE)에서는 TSP-1의 발현을 감소시키는 반면 사람 간암 세포주인 Hep3B에서는 증가시키는 사실을 발견하였다. PMA 처치에 따른 정상 돼지 대동맥 내피세포에서의 TSP-1의 발현 감소현상은 *tsp-1* 유전자 조절부위의 염기서열 -767과 -723 사이에 존재하는 염기서열이 억제 부위임을 밝혀 이러한 결과를 바탕으로 -767에서 -723 염기서열을 서로 부분 중복되도록 세 종류의 올리고 탐식자 (올리고 탐식자 a-1, -767~-738; 올리고 탐식자 a-2, -759~-730; 올리고 탐식자 a-3, -752~-723)를 제작하여 -767과 -723 부위의 특정 염기서열과 이에 결합하는 인자를 EMSA를 수행하여 분석하였다. 실험 결과, PMA 처치에 따른 정상 돼지 대동맥 내피세포에서의 TSP-1 감소는 -752에서 -730 사이의 염기서열이 저해 조절인자와 결합함과 더불어 -767에서 -760과 -752에서 -730 사이의 염기서열들에 촉진 조절인자들이 결합하지 못함으로써 기인된다는 실험적 사실을 관찰하였고. 특히, PMA 처치는 정상 돼지 대동맥 내피세포에서 저해 조절인자의 -752에서 -730 부위에 대한 친화력을 향상시켰으며 이러한 친화력은 c-Jun 항체에 의해 영향을 받지 않았다.