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Ets-1 Upregulates Matrix Metalloproteinase-1 Expression through Extracellular Matrix Adhesion in Vascular Endothelial Cells

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Ets-1 is a transcription factor regulating the expression of matrix-degrading proteinases and is believed to play a critical role in cell migration and tumor invasion. The aim of this study is to investigate the direct induction of ets-1 with consequential upregulation of collagenase-1 (MMP-1) by cell adhesion to extracellular matrix and to identify intracellular signal transduction pathways involved in ets-1 induction in cultured endothelial cells. The expressions of ets-1 mRNA and protein as well as MMP-1 protein were induced by cell adhesion to type I collagen and antisense ets-1 oligonucleotides impaired that MMP-1 expression. In addition, protein tyrosine kinase (PTK) and protein kinase C (PKC) inhibitors abrogated their induction, showing the suppression of focal adhesion kinase phosphorylation. These results suggest that ets-1 induced by cell adhesion to extracellular matrix directly upregulates MMP-1 expression via PTK and PKC activation in cultured endothelial cells.

Key Words: **ets-1; MMP-1; type I collagen; endothelial cell; FAK; β1-integrin.**

Vascular smooth muscle cells (VSMC) and endothelial cells (EC) play a key role in the formation of the hyperplastic response to arterial injury including interventions for occlusive vascular disease (1). These events are histopathologically observed by migration of VSMC and EC into the intima with controlled secretion of extracellular matrix components such as collagen, laminin and fibronectin (2), and extracellular matrix metalloproteinases (MMPs) such as collagenase-1 (MMP-1) and stromelysin (MMP-3) (3, 4). On the other hand, a similar process is observed in the migration of EC from pre-existing capillaries with controlling matrix metalloproteinase in the formation of a new capillary plexus in remodeling tissues (5). As cell migration is a multistep process that involves adhesion, motility and degradation of the extracellular matrix, it seems reasonable to suppose that the migratory cell may be continuously exposed to the adhesive stimulation caused by contact between cell and extracellular matrix and be regulated by it during the tissue remodeling process. In fact, Elsdale (6) and Bell *et al.* (7) have demonstrated that the extracellular matrix such as collagen has influence upon cell form, motility, adhesion and growth in fibroblast *in vitro.*

The ets-1 gene is known to encode a transcription factor that activates expression of MMP-1, MMP-3 and urokinase-type plasminogen activator (uPA) through the ETS binding site which contains a central GGAA/T core motif (8–10). Recently, it has been reported that ets-1 takes part in angiogenesis, regulating the expression of extracellular matrix metalloproteinases and the migration of EC (11). In addition, we have reported that ets-1 is an early response gene induced in cultured VSMC through protein kinase C (PKC) and/or protein tyrosine kinase (PTK) activation by several mitogens and chemotactic factors known to participate in VSMC migration and proliferation (12, 13). These previous reports lead us to hypothesize that ets-1 may play an important role in the biological process between cell adhesion to extracellular matrix and migration. However, it is not yet clear whether the stimulation of cell adhesion to extracellular matrix directly induces ets-1 gene expression in a cell. This study, therefore, aims to

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determine whether there is induction of ets-1 and MMP-1 by cell adhesion to the extracellular matrix component type I collagen, to investigate the direct interaction between ets-1 and MMP-1 in this process, and furthermore to explore the involvement of intracellular signal transduction pathways for ets-1 expression by using specific protein kinase inhibitors.

MATERIALS AND METHODS

Cells and cell culture. Bovine aortic endothelial cells were cultured as previously described (14). Fresh bovine thoracic aortae obtained from an abattoir were kept in ice-cold phosphate-buffered saline (PBS) solution containing 100 U/ml penicillin and 100 μg/ml streptomycin. Endothelial cells were obtained by scraping the luminal surface with a razor blade and were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 in air. Endothelial cells were characterized as previously described (14). First, in this study, these endothelial cells were treated with PMA and endothelin-1 to examine the ets-1 mRNA induction.

Cell adhesion to Type I collagen. Forty-eight hour serum-deprived EC after growing to about 80% confluence in DMEM/F12 with 10% FBS were stripped from dishes by 0.25% trypsin–0.02% EDTA in PBS and these cells were sparsely seeded into a 75-cm^2 plastic dish (Coster Corp., Cambridge, MA; 1×10^4 cells/dish) with medium (DMEM/F12) containing 0.1% FBS. Half of the dish area was thinly coated with Type I collagen gel while the other half was uncoated. Type I collagen gel solution (0.5 ml) consisted of a mixture of 8 vol of type I collagen solution, 1 vol of 10× DMEM/F12, and 1 vol of 0.05 N NaOH, 200 mM Hepes, and 260 mM $NaHCO_3$. Type I collagen gel solution was incubated for 60 min at 37°C for coating to the plastic dish.

Reagents. Endothelin-1, phorbol 12-myristate 13-acetate (PMA), herbimycin A, and H-7 were purchased from Sigma Chemical Co. (St. Louis, MO). Type I collagen solution was purchased from Koken (Tokyo, Japan).

cDNA probe. The following cDNA probe was used: The rat ets-1 probe was a 1.4-kb *Bam*HI fragment of ets-1 cDNA cloned in the pLXSN plasmid vector. The probe was gel-purified and ^{32}P-labeled by random priming as described previously (15).

Oligonucleotide transfection. Phosphorothioate oligodeoxy nucleotides were synthesized using a Milligen Biosearch Cyclone Plus programmable synthesizer. The oligodeoxynucleotides were further purified on a TSK gel Oligo-DNA RP (4.6 mm interior diameter × 15 cm) high-performance liquid chromatography column. The oligonucleotides used in this study were 20-mer in length and directed to the region of translation initiation of the corresponding ets-1 mRNA. The following ets-1 antisense, sense, and mismatch were used: ets-1 antisense, 5′>AGA TCG ACG GCC GCC TTC AT<3′; sense, 5′>ATG AAG GCG GCC GTC GAT CT<3′; mismatch, 5′>AGA TCG CTG GAC GCC TTC AT<3′. EC were treated with these oligonucleotides as described by Bennett *et al.* (16). Briefly, after EC were grown to about 80% confluence in DMEM/F12 with 10% FBS, 48 h serum-deprived EC were stripped from the dish by 0.25% trypsin–0.02% EDTA in PBS. These cells were seeded in a 75-cm^2 plastic dish (Coster Corp.; 1×10^4 cells/dish) coated with Type I collagen in serum-free medium (DMEM/F12) including 20 μM oligonucleotides for 8 h.

Northern blot analysis. Total RNA was extracted from cultured bovine endothelial cells with ISOGEN Reagent (Nippon Gene, Co. Ltd., Toyama, Japan). Twenty micrograms of total RNA were size separated by electrophoresis through 1% agarose–formaldehyde gels. RNA samples were transferred to nylon membranes (Micron Separation Inc., Westborough, MA), and hybridized to the indicated random prime-labeled cDNA probe. Hybridization reactions were carried out for 16 to 24 h at 65°C in 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 1% BSA, 7% sodium dodecyl sulfate (SDS) and 30% formamide. Membranes were washed in 20 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA (pH 8) and 1% SDS and exposed to Kodak X-omat AR film at −80°C for 12 to 72 h.

Immunohistochemistry. Cultured EC were fixed with 4% paraformaldehyde in 0.01 M phosphate buffer (PB) (0.33 g NaH_2PO_4 $2H_2O$ + 2.87 g Na_2HPO_4 $12H_2O$/L) for 10 min after removal of medium and were then washed with PBS three times. They were preincubated with normal goat serum to prevent nonspecific binding after 3% H_2O_2 treatment for 10 min and were incubated with antibodies as follows: 0.1 μg/ml anti-human Ets-1 (C-20, raised against the carboxyl-terminal domain of Ets-1 protein; Santa Cruz Biotechnology, Santa Cruz, CA), anti-human MMP-1 antibody (anti-hMMP-1, purified IgG; Fuji Chemical Industries, Ltd., Takaoka, Japan), anti-β1 integrin antibody (formalin grade, Funakoshi, Japan) and anti-human ICAM-1 antibody (Wako, Japan). They were detected by the avidin–biotin–peroxidase complex method (17) with 3-amino-9-ethylcarbazole-hydrochloride (AEC) colorization for ets-1 and β1 integrin, and DAB colorization for MMP-1 and ICAM-1.

Western blot analysis. At 8 h after seeding to a dish coated with Type I collagen, EC were washed twice in ice cold PBS and lysed in a lysis buffer [20 mM Tris–HCl (pH 7.4), 0.1% Triton X-100, 1% sodium deoxycholate, and 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (PMSF)]. The cell lysates were incubated for 30 min followed by centrifugation at 15,000 rpm for 10 min at 4°C, and after collecting the supernatants, the protein concentration was determined by the BCA protein assay regent (Pierce, Rockford, IL). Samples containing an equivalent amount (20–50 μg) diluted with SDS sample buffer [2% SDS, 25 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, and 10% glycerol] were boiled for 5 min, applied on 10% polyacrylamide gels containing 0.1% SDS and electrophoresis was carried out. Separated proteins were electroblotted onto a Hybond-N nitrocellulose membrane (Amersham, Arlington Heights, IL). Nonspecific binding was blocked with blotto-Tween (5% nonfat milk, 0.05% Tween 20 and 50 mM Tris, pH 7.5), incubated with mouse anti-chicken FAK monoclonal antibody (Transduction Laboratories, Lexington, KY) followed by the horseradish peroxidase conjugated anti-mouse IgG (Amersham, Buckinghamshire, UK). The membranes were developed according to Amersham's ECL protocol. Finally, an anti-phosphotyrosine detection kit (nano Tools Antikorpertechnik, Teningen) was used for analysis of phosphorylation of tyrosine residues in FAK.

Density analysis. Abundance of ets-1 and MMP-1 protein in Fig. 1b and FAK and phosphorylated FAK in Fig. 4 was calculated by densitometry with NIH image.

RESULTS

Effects of Endothelin-1 and PMA on ets-1 mRNA Level

We first investigated ets-1 gene induction by endothelin-1- and PMA-stimulation in cultured EC to confirm whether similar results already observed in VSMC (12) are obtained in EC. A significance of Fig. 1a is to check the reactivity of cultured bovine aortic endothelial cells to endothelin-1 and to confirm the cross-reactivity of rat ets-1 probe in these endothelial cells, although those were already checked in the previous studies (14, 25). Endothelial cells at about 80% confluency were cultured with DMEM/F12 containing 10%

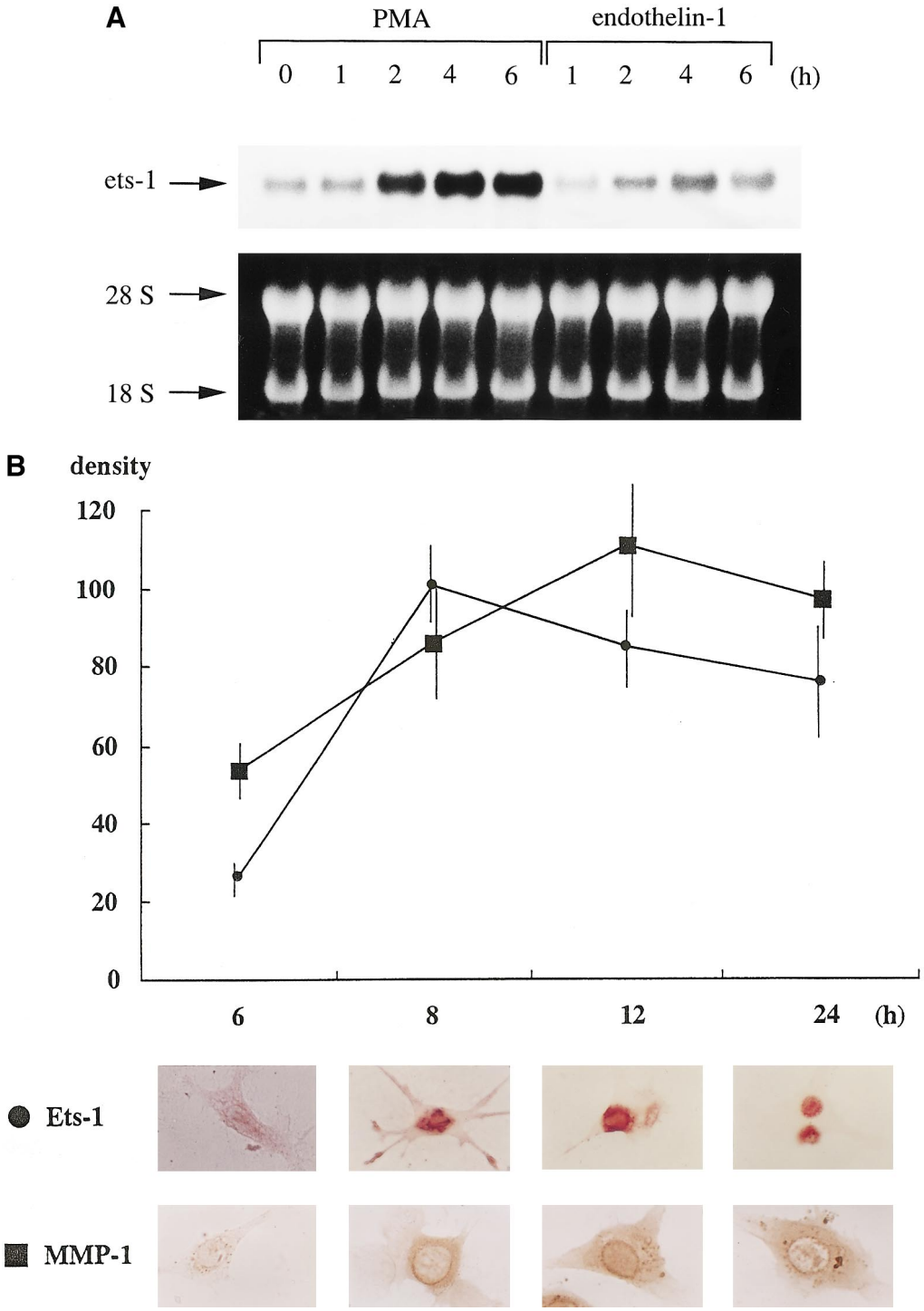

FIG. 1. (A) Time course for the induction of ets-1 mRNA by PMA and ET-1. EC were serum starved for 48 h and then treated with PMA (100 ng/ml) and ET-1 (100 ng/ml) for indicated times. Representative Northern blot of 20 μg RNA was sequentially hybridized to the ets-1 cDNA probe. Arrows point to ets-1 mRNA transcripts of 5.3 kb and ethidium bromide staining of ribosomal RNA bands. (B) Sequential induction of Ets-1 and MMP-1 proteins by cell adhesion to Type I collagen gel. Abundance of Ets-1 (●) and MMP-1 (■) proteins was calculated by densitometry with NIH image.

FBS and then starved with 0.1% FBS for 48 h. The serum-deprived EC were treated with PMA (100 ng/ml) and endothelin-1 (100 ng/ml), respectively. As shown in Fig. 1, endothelin-1 and PMA induced significant expression of ets-1 mRNA transcript of 5.3 kb at 2 ~ 4 h after exposure.

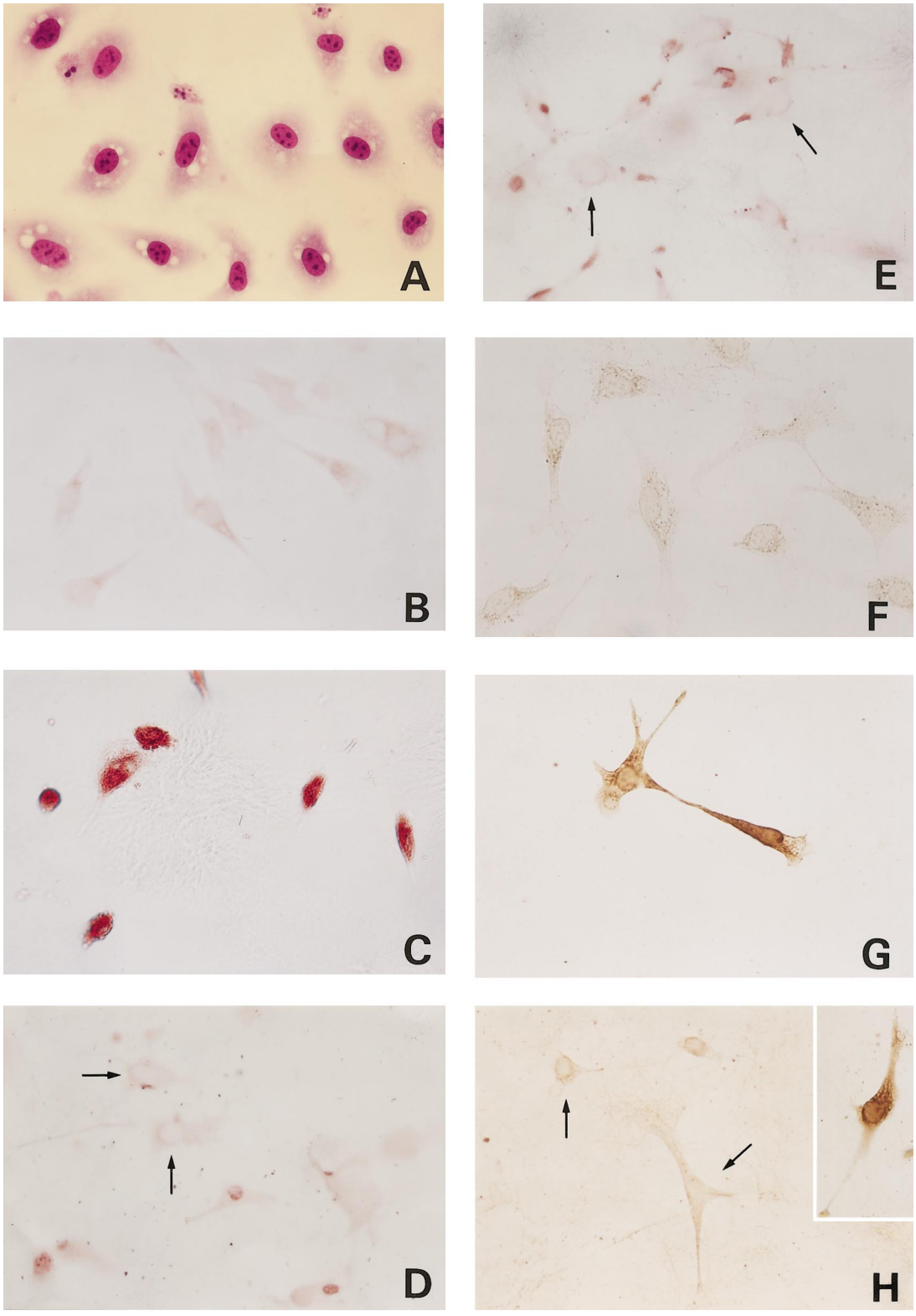

FIG. 2. Ets-1 and MMP-1 protein expression in Type I collagen coated dish. (A) Endothelial cells in normal condition (Giemsa stain, ×200). (B) Ets-1 expression in EC spread for 8 h at 37°C with DMEM/F12 medium containing 0.1% serum on an uncoated dish. (C) Ets-1 expression in EC spread for 8 h at 37°C with serum free medium (DMEM/F12) on Type I collagen-coated dish. Strong expression of ets-1 protein was observed in the nucleus of EC. (D) Ets-1 expression on Type I collagen coated dish under the treatment with H-7 (20 μM). (E) Ets-1 expression on Type I collagen coated dish under the treatment with herbimycin A (0.75 μg/ml). H-7 and herbimycin A inhibit ets-1 expression (arrow). (F) MMP-1 protein expression in EC spread for 8 h at 37°C with DMEM/F12 medium containing 0.1% serum on an uncoated dish. (G) Strong expression of MMP-1 protein in EC spread for 8 h at 37°C with DMEM/F12 medium containing 0.1% serum on Type I collagen coated dish. (H) MMP-1 protein expression in EC spread on Type I collagen gel with antisense ets-1 oligonucleotides. Antisense ets-1 oligonucleotides strongly inhibit MMP-1 protein expression (arrow). Inset shows that sense ets-1 oligonucleotides have no effect on MMP-1 protein expression. (B, C, D, E: AEC colorization for Ets-1 immunostain, ×200)/(F, G, H: DAB colorization for MMP-1 immunostain, ×200).

Effect of Cell Adhesion to Type I Collagen on ets-1 and MMP-1 Protein Levels

Figure 1b shows the time course of induction of Ets-1 (●) and MMP-1 (■) proteins after sparsely cells' seeding on Type I collagen gel. The expression of ets-1 protein was maximal at 8 h and that of MMP-1 was maximal at around 12 h. The expression levels of Ets-1 were gradually decreased and returned to basal levels after 8 h (data not shown). However, high expression levels of MMP-1 were still maintained after 12 h. Ets-1 was mainly expressed in the nucleus and MMP-1 was in the cytoplasm of endothelial cell.

Effects of Herbimycin A and H-7 on the ets-1 and MMP-1 Protein Expression Induced by Cell Adhesion to Type I Collagen

Inhibitors specific for PTK or PKC are useful tools for understanding the role of PTK or PTK in signal transduction pathways involved in cell proliferation or differentiation. Herbimycin A is a well characterized inhibitor of PTK. H-7 inhibits PKC activity via a direct interaction on the catalytic site of the enzyme and suppresses PKC-mediated phosphorylation. Forty-eight-hour serum-deprived ECs were stripped from a dish by 0.25% trypsin–0.02% EDTA in PBS and these cells were sparsely seeded in a 75-cm^2 plastic dish coated with Type I collagen gel in medium (DMEM/F12) containing low serum (0.1% FBS) including 20 μM H-7 or 0.75 μg/ml herbimycin A. Ets-1 protein expression induced by cell adhesion to Type I collagen with treatment of H-7 or herbimycin A was remarkably diminished at around 8 h after seeding, compared with that without H-7 or herbimycin A treatment (Figs. 2D and 2E). In addition, MMP-1 protein expression was also inhibited by the treatment with H-7 and herbimycin A (data not shown). Addition of herbimycin A or H-7 at the above concentration did not affect cell viability. H-7 and herbimycin A is though to have broad spectrum. Then, we furthermore examined by using other protein kinase inhibitors such as 10 nM staurosporine (PKC inhibitor, Sigma, Tokyo, Japan) and 30 μM genistein (PTK inhibitor, Sigma), although they also have relatively broad spectrum as staurosporine is inhibitor of phospholipid/calcium-dependent protein kinase and genistein is inhibitor of protein tyrosine kinase; competitive inhibitor of ATP in other protein kinase reactions. They also attenuated the expression of ets-1 and MMP-1 proteins as well as H-7 and herbimycin A (data not shown). The inhibitors at the concentration used were not toxic for cells.

Effect of Cell Adhesion to Type I Collagen on ets-1 mRNA Levels

To examine the ets-1 mRNA induction, 48 h serum-deprived endothelial cells were stripped from a dish by 0.25% trypsin–0.02% EDTA in PBS and these cells were sparsely seeded into a 75-cm^2 plastic dish coated with Type I collagen gel containing low serum (0.1% FBS) medium only or medium including 20 μM H-7 or 0.75 μg/ml herbimycin A. Total RNA was extracted with ISOGEN Reagent 8 h after seeding. Twenty μg of total RNA was used for Northern blot hybridization as described under Materials and Methods. Figure 3 showed that ets-1 mRNA expression of cells in the dish coated with Type I collagen (lane 2) is stronger than that of the control (lane 1). Forty-eight-hour serum-deprived cells without treatment were used as control. H-7 (lane 4) and herbimycin A (lane 5) strongly inhibited ets-1 mRNA expression observed in lane 2. Cell handling such as stripping and seeding weakly induced ets-1 mRNA expression (lane 3).

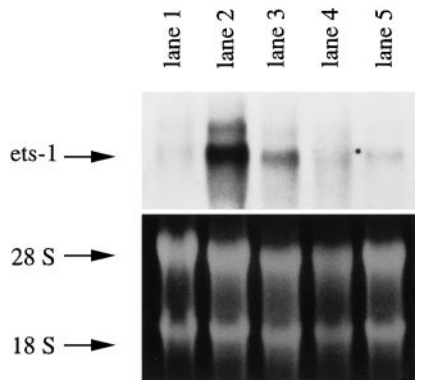

FIG. 3. Ets-1 mRNA expression induced by cell adhesion to Type I collagen. Forty-eight-hour serum-deprived endothelial cells were stripped from the dish by 0.25% trypsin–0.02% EDTA in PBS and these cells were sparsely seeded into a 75-cm^2 plastic dish coated with Type I collagen gel containing serum free medium and harvested for RNA extraction at 8 h after spreading. Representative Northern blot of 20 μg RNA was sequentially hybridizes to ets-1 cDNA probe. Arrows point to ets-1 mRNA transcripts of 5.3 kb and ethidium bromide staining of ribosomal RNA bands. Lane 1, 48-h serum-deprived EC; lane 2, EC spread on Type I collagen gel; lane 3, EC spread on an uncoated dish; lane 4, EC spread on Type I collagen gel with H-7 (20 μM) treatment; lane 5, EC spread on Type I collagen gel with herbimycin A (0.75 μg/ml) treatment.

Effect of Antisense ets-1 Oligonucleotides on MMP-1 Induced by Cell Adhesion to Type I Collagen

Treatment with antisense oligonucleotides to cultured cells is often useful for the observation of its effect on the target gene and its protein expression. Antisense ets-1 oligonucleotides strongly inhibited the expression of Ets-1 protein (data not shown) and MMP-1 protein induced by cell adhesion to Type I collagen (Fig. 2H). However, ets-1 sense and mismatch oligonucleotides gave no effect (Fig. 2H, inset: ets-1 sense).

FAK and Its Tyrosine Phosphorylation Induced by Adhesion to Type I Collagen

Western blotting showed that the antibody specific for FAK recognized a single band (125 kDa) with al-

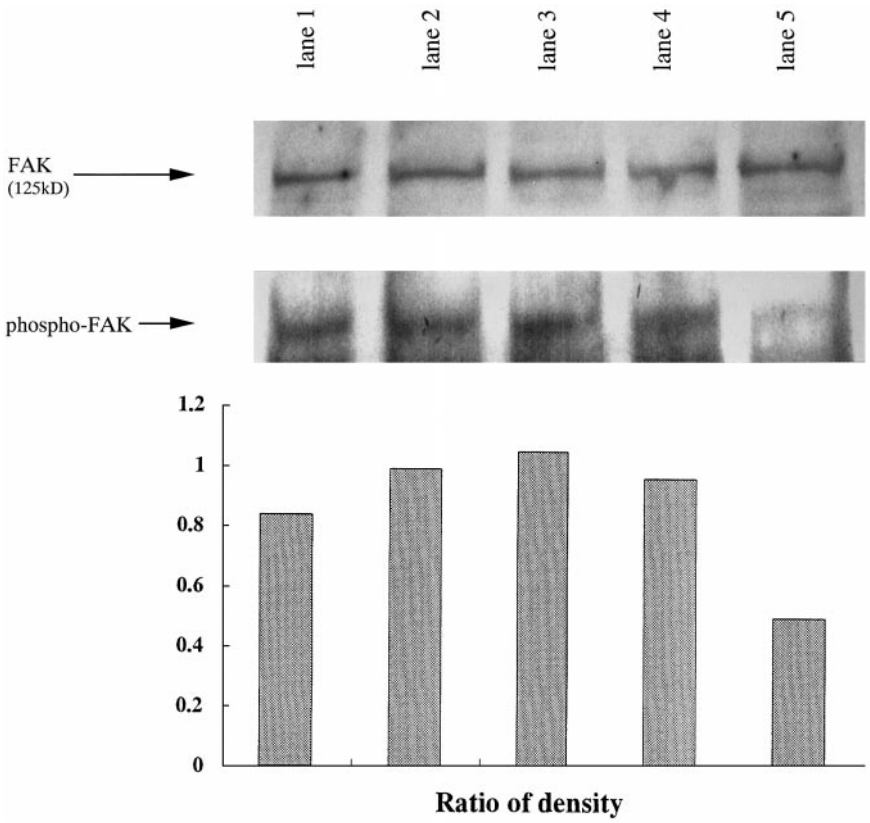

FIG. 4. FAK expression and its phosphorylation. Western blotting showed that the antibody for FAK recognized a single band (125 kDa) with almost the same volume in all samples respectively and the antibody for tyrosine phosphorylation recognized a single band at the location of the FAK molecular weight (125 kDa). Lane 1, 48-h serum-deprived EC; lane 2, EC spread on an uncoated dish; lane 3, EC spread on Type I collagen gel; lane 4, EC spread on Type I collagen gel with H-7 (20 μM) treatment; lane 5, EC spread on Type I collagen gel with herbimycin A (0.75 μg/ml) treatment.

most the same volume in all samples respectively and the antibody specific for tyrosine phosphorylation recognized a single band at the location of the FAK molecular weight (125 kDa) (Fig. 4). The individual lane in Fig. 4 shows as follows: lane 1, 48-h serum-deprived EC; lane 2, EC spread on an uncoated dish; lane 3, EC spread on Type I collagen gel; lane 4, EC spread on Type I collagen gel with H-7 (20 μM) treatment; lane 5, EC spread on Type I collagen gel with herbimycin A (0.75 μg/ml) treatment. Although the intensity in lanes 2, 3, and 4 seems to be slightly increasing in the density graph, there are no remarkable differences among lanes 1, 2, 3, and 4. However, tyrosine phosphorylation of FAK in cells treated with herbimycin A (lane 5) was obviously decreased compared with others although tyrosine phosphorylation of FAK in other lanes were not remarkably different.

β1 Integrin and ICAM-1 Expression in Endothelial Cells Adhering to Type I Collagen

Figure 5 showed that β1 integrin and intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells adhering to Type I collagen. Integrins are known to promote highly stabilized adhesion to extracellular matrix proteins and endothelial cells are known to use β1 integrin to attach on collagen (18). ICAM-1 is adhesion molecule belonging to immunoglobulin family and ligand for leukocyte function associated antigen-1 (LFA-1) (19). ICAM-1 does not contribute to attachment on collagen. β1 integrin expression in endothelial cells seemed to be slightly increased in Type I collagen coated dish (Fig. 5B) compared to β1 integrin control (Fig. 5A). However, ICAM-1 expression seemed to be unchanged compared to the ICAM-1 control (Figs. 5C and 5D). We repeated this immunohistochemical study three times and similar staining results were observed.

DISCUSSION

Cell migration and tumor invasion that occur in various physiological and pathological processes involving adhesion, motility and degradation of extracellular matrix. Ets-1 plays an important role in them via regulating extracellular matrix metalloproteinases such as MMP-1 and MMP-3. Although ets-1 is known to be

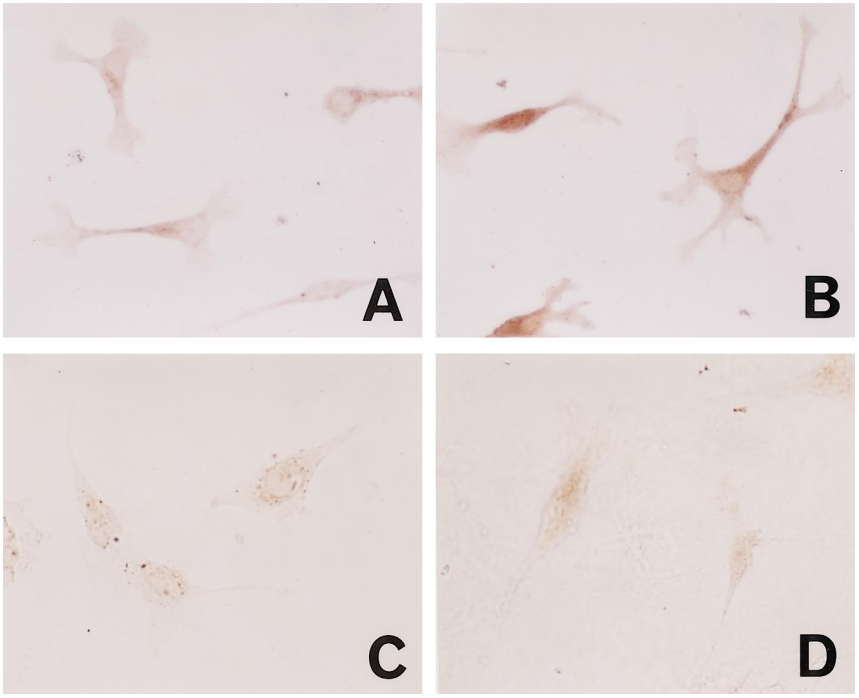

FIG. 5. β1 integrin and MMP-1 expression in endothelial cells adhering to Type I collagen. (A) β1 integrin expression seemed to be slightly increased compared to the β1 integrin control (B) although it was not remarkable. (C) ICAM-1 expression seemed to be unchanged compared to the ICAM-1 control (D). This immunohistochemical study was performed three times and the same staining results were observed.

induced by some growth factors and chemotactic factors such as endothelin-1, PDGF-BB as well as PMA in cultured VSMC (12, 13), it is still unknown whether mechanical stimulation such as cell contact to extracellular matrix can induce ets-1. In the present study, we provided evidence of the direct induction of ets-1 mRNA and protein by cell adhesion to type I collagen in EC. Furthermore, our data demonstrated that ets-1 mRNA expression could also be induced by cell handling including stripping and seeding onto plastic dishes as shown in the lane 3 of Fig. 3. It has been reported that mechanical stimulation such as shear stress derived from blood pressure on the vascular wall induces various signals for vascular remodeling (22), and a three-dimensional collagen lattice activates DNA binding activity of the nuclear transcription factor (NF-κB) in human fibroblasts (23). These findings suggest that physical stimulation and some growth factors are capable of inducing these transcription factors through various intracellular signal transduction pathways, which matches our present results.

MMPs are active in remodeling fibrous connective tissue and one of their roles is considered to allow cells to move into extracellular matrix following their degradation of it. We revealed that MMP-1 and Ets-1 was induced by adhesion to type I collagen and that ets-1 directly regulated MMP-1 induction as demonstrated by antisense ets-1 oligonucleotide inhibition. Regarding the effect of antisense ets-1 oligonucleotide used in this study, our recent report has shown that it strongly inhibits the invasiveness of the glioblastoma cell line T98G through the down regulation of uPA induction, which along with MMP-1 is one of the important matrix degrading proteinases (24), and blocks H_2O_2-induced angiogenesis through the inhibition of EC tube formation (25). These findings permit us to speculate that cell adhesion to extracellular matrix component such as type I collagen induces MMP-1 through the transcriptional function of ets-1.

Tissue inhibitors of metalloproteinases (TIMPs) are also important factors involved in cell migration and tumor invasion (26) although we did not examine them in this study. It has been reported that TIMPs are induced by cell adhesion to extracellular matrix and the activation of matrix metalloproteinase is reduced by TIMPs in melanoma cells (27, 28). Cell migration and tumor invasion might be performed as a balance between degradative and formative mechanisms for extracellular matrix through the activation and/or inactivation of MMPs and TIMPs. The interactions between ets-1 and TIMPs are still relatively unknown, but a few have been reported (29). To understand the cell migratory mechanism, we will further examine the interaction among ets-1, MMPs and TIMPs.

With respect to the signal transduction pathways acting in transmitting signals from growth factor-, cytokine-, and hormone-receptor interactions, major components of these signal transduction pathways are

protein kinase A (PKA), protein kinase C (PKC) and protein tyrosine kinase (PTK) (21, 30, 31). Therefore, the respective protein kinase inhibitor is useful in defining a direct or indirect role for specific kinases in signal transduction induced by various stimulating factors. Using them, we have previously revealed that PKC and/or PTK activation and intracellular Ca^{2+} stored in the endoplasmic reticulum are pivotal components of the signal transduction pathway for activation of ets-1 gene expression by endothelin-1 (ET-1) and PDGF-BB in cultured VSMC (12). In this study, we showed that PKC and PTK activation after cell adhesion to Type I collagen were key components for the induction of ets-1 and MMP-1, and that FAK might be a PTK involved in this process. There are some reports about the interaction between PKC activation and FAK phosphorylation, one of which has shown that activation of PKC by adding phorbol ester to $\alpha v\beta 5$-expressing melanoma cells triggers tyrosine phosphorylation of FAK (32). However, a signaling process from PKC to FAK seems to be absent in the present study because PKC inhibitor had no effect on FAK phosphorylation as shown in Fig. 4. Schaller (33) and Ilic *et al.* (34) have showed that the $\beta 1$ integrin subunit can bind directly to FAK and the biological function of integrin is exercised through FAK activation. In addition, collagen and vitronectin act as ligands for adhesion molecules such as $\beta 1$ and/or $\beta 3$ integrins for spreading and migration of endothelial cells (18). Indeed, the expression of $\beta 1$ integrin in EC seems to have immunohistochemically increased after adhesion to type I collagen, and the expression of ICAM-1 without contribution to attachment on collagen was unchanged as shown in Fig. 5, which suggests that increase on $\beta 1$ integrin expression may be specific reaction for collagen. $\beta 1$ integrin binding might be involved in the adhesion mechanism which initiates the signal transduction pathway for ets-1 expression. It has been reported that adhesion molecules such as integrins interact with the extracellular matrix involving $p125^{FAK}$(FAK) tyrosine phosphorylation which plays an important role in cytoskeletal assembly when cells adhere to the extracellular matrix (35–37). Considering that ets-1 participates in cell differentiation and migration regarding cytoskeletal rearrangement, it is easy to conclude that these adhesion molecules are present upstream of the signal transduction pathway for ets-1 expression. The cell adherence to type I collagen via $\beta 1$ integrin binding directly leads to FAK phosphorylation and may directly or indirectly activate PKC for ets-1 induction. On the other hand, the present results as cell attachment to type I collagen did not induce a clear upregulation of FAK phosphorylation shown in Fig. 4 may indicate the significance of other tyrosine kinases such as src family for the signal transduction pathway of ets-1 induction. And this speculation may be supported by reports showing that Src as well as FAK is an important molecule in the signal transduction pathways by cell adhesion (40, 41).

In the formation of the hyperplastic response to arterial injuries such as arteriosclerosis and experimental arterial balloon injury, the first stimulus for cellular migration into the intima and proliferation of intimal cells to form a thickened neointima is from some mesenchymal cell growth factors such as PDGF-BB released from platelets at the injury site, as was originally proposed by Ross (38) and Jawien (39). The second stimulus might be cellular adhesion to the surrounding extracellular matrix and/or from cytokines released from aggregating inflammatory cells, in addition to mechanical stress by blood flow in the injured vascular lumen. The expression of Ets-1 transcription factor with consequential MMP-1 upregulation in migratory cells during the vascular remodeling process may be induced and maintained by these stimuli, and play a crucial role in cell migration.

In conclusion, the present results demonstrate that Ets-1 transcription factor induced through PKC and PTK activation after cell adhesion to type I collagen directly upregulates MMP-1 expression.

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