Original Article

Suppression of Integrin α_v Expression by Endothelin-1 in Vascular Smooth Muscle Cells

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Both integrins and endothelins (ETs) are known to play important roles in vascular remodeling *via* proliferation, apoptosis, and migration of vascular smooth muscle cells (VSMCs), whose dysfunctions have been implicated in the pathogenesis of end-organ damage associated with hypertension and arteriosclerosis. However, whether there is any interaction between endothelin-1 (ET-1) and integrins remains unknown. Therefore, the aim of the present study was to elucidate whether ET-1 regulates the expression of integrin α_v in rat VSMCs. ET-1 dose- and time-dependently suppressed the integrin α_v messenger RNA (mRNA) transcripts, as quantified by a real-time quantitative polymerase chain reaction (PCR) method, and decreased the transcriptional activity of integrin α_v gene, as demonstrated by integrin α_v -luciferase assay. The inhibitory effect of ET-1 on integrin α_v gene expression was abrogated by an ET_A receptor antagonist (BQ123) but not by an ET_B receptor antagonist (BQ788). ET-1 also suppressed the cell surface expression of integrin $\alpha_v\beta_5$ and the adhesion to vitronectin, but not to fibronectin. These results demonstrate that the adhesion of vitronectin to rat VSMCs is inhibited by ET-1 *via* the ET_A receptors by suppressing integrin α_v gene transcription, suggesting that ET-1 is involved in regulation of vascular integrin α_v gene expression. (*Hypertens Res* 2000; 23: 643-649)

Key Words: endothelin-1, integrin α_v , vitronectin, vascular smooth muscle cells

Introduction

Adhesion molecule receptors and their ligands together provide cells with anchorage and traction for migration, while the receptors mediate signals controlling cell proliferation, survival, differentiation and expression of genes (1). Integrins are a major group of versatile adhesion receptors that serve both adhesive and signaling functions. For example, the binding of VSMCs to vitronectin is mediated by members of the integrin α_v family; that is, vitronectin significantly enhances the migration of vascular smooth muscle cells (VSMCs) *via* the integrin $\alpha_v\beta_3$ re-

ceptors (2-4). The activation of the integrin $\alpha_v\beta_3$ receptors is an important determinant of insulin-like growth factor (IGF)-I-mediated migration (5). Thus, the migration of VSMCs is one of the most important initial events contributing to vascular remodeling. In the event of mechanical or other endothelial injuries, such as hypertension, arteriosclerosis, and post-angioplasty restenosis, VSMCs normally quiescent in the tunica media proliferate and migrate into the intima and contribute to the narrowing of vessels (6). This process is triggered and regulated by multiple factors, including cytokines, peptide growth factors, integrins, and specific components of the extracellular matrix (6, 7).

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Endothelin (ET)-1, a potent vasoconstrictive peptide produced by and secreted from endothelial cells, elicits its actions on VSMCs mainly by binding to the ET_A receptors. It has been reported that an enhanced ET-1/ET_A receptor system is involved in the development of end-organ damages in DOCA-salt-induced hypertension (8), left ventricular hypertrophy induced by nitric oxide synthase inhibitors (9) and neointimal formation after balloon injury (10). In spite of the diverse actions of ET-1, including mitogenesis of VSMCs (11, 12), apoptosis survival (13, 14), and migration of endothelial cells (15), the role of ET-1 in the migration of VSMCs is less well characterized. Recently, it has been shown that ET-1 stimulates VSMC migration induced by lysophosphatidylcholine *in vitro* (16). Growth factors such as transforming growth factors and IGFs have been shown to modulate integrin expression on cell surfaces of VSMCs, but whether ET-1 modulates adhesion molecules remains undetermined.

These observations prompted us to test whether ET-1 modulates the expression of integrin α_v , a major component of vitronectin receptor essential to cell proliferation and migration, in cultured rat VSMCs.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow Lab., fetal calf serum (FCS) was purchased from Cell Culture Lab., and synthetic ET-1 was purchased from the Peptide Inst., Inc. ET_A receptor antagonist, BQ123, ET_B receptor antagonist, BQ788, and ET_B receptor agonist, BQ3020, were generously supplied by Banyu Research Lab. U73122, GF109203X, LY294002, and ST638 were purchased from Calbiochem-Novabiochem Int. (CA), and nicardipine, and wortmannin were purchased from Sigma-Aldrich, Japan (Tokyo). All other compounds or reagents were of molecular biology grade.

Cell Culture

Rat aortic VSMCs were prepared by the explant method as reported previously (17, 18) and cultured in DMEM supplemented with 10% FCS in 5% CO₂ atmosphere at 37°C, and then the cells (6–12th passages) were used in the experiments. The smooth muscle origin was confirmed by staining with mouse monoclonal antibody against anti-smooth muscle actin (Lab Vision Corp., Fremont, USA).

Real-Time Quantitative Polymerase Chain Reaction (PCR)

A real-time quantitative PCR method was used to detect the changes of rat integrin α_v gene copies. The cycle at

which the amplification plot crosses the threshold (C_T) is known to accurately reflect relative messenger RNA (mRNA) values (19). Total RNA was harvested from cells treated with or without ET-1 or indicated reagents. Rat integrin α_v was amplified (forward primer 5'-GCAA GACTGTCCTGTGCATTTAAG-3' and reverse primer 5'-AGTTGAGTTCCAGCCTTCTTCG-3'), detected, and quantified in real time using the ABI Prism™ 7700 Sequence Detection System (Applied Biosystems, Foster City, USA) essentially as described (20). The TaqMan probe was 5'-AGAAAACCAAACCCGGCAGGTGGT-3'. The amplification mixture contained 5 nM of template DNA and 50 μ M of primer DNA in 50 mM salt and 1 mM Mg²⁺. A three-step PCR was performed for 35 cycles. Denaturation was done at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. The reaction produced an 88-base pair (bp) PCR product. The PCR cycle at which the amplification plot crosses a threshold of 10 standard deviations above the baseline was defined as the C_T value. In comparison to a control sample without RNA, which did not show any increase in normalized reporter signal (R_n), the addition of mRNA extracted from rat VSMCs resulted in PCR amplification reaching the threshold value. The relative mRNA level was calculated based on the assumption that C_T values increase by approximately one for each two-fold dilution (19). To determine the precision of the assay, integrin α_v mRNA from VSMCs were reverse transcribed and amplified on four separate days. The mean C_T values ranged from 25.78 to 28.39, and the result was an intra-assay precision from 0.49% to 0.78% coefficient of variation. The inter-assay precision of amplification for the 4 days was 4.2%.

Construction of Luciferase Fusion Genes and Transient Gene Expression Assays

pGL3-Basic Vector (Promega, Madison, USA) was used to make reporter constructs containing mouse integrin α_v promoter sequence that will drive luciferase gene expression. PCR-amplified DNA fragments of 1,164 bp consisting of the mouse integrin α_v and additional modified nucleotides were digested with Kpn I and Sac I, and the resulting 1,140-bp fragment spanning -1,137 to +3 plus sticky ends were subcloned into the Kpn I/Sac I site of pGL3-Basic. The integrity of the construct was verified by sequencing. For transfection, VSMCs were seeded into 96-well plates, and, after 24 h, a DNA mixture containing 1 μ g of integrin α_v reporter construct and 0.1 μ g of pRL-TK vector (Promega) expressing renilla luciferase were transfected into VSMCs with a transferrin receptor-operated transfer method (21) with modification in order to further improve transfection efficiency (13, 20). In brief, 3 μ l of synthetic cationic liposome, (+)-N,N-bis(2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide (Promega), was added to 100 μ l HEPES-

Fig. 1. Down-regulation of integrin α_v mRNA by ET-1 as detected by real-time quantitative PCR. Cells were treated with the indicated concentrations of ET-1 for 6 h in combination with an ET_A receptor antagonist, BQ123 (10^{-6} M) or an ET_B receptor antagonist, BQ788 (10^{-6} M). Extracted total RNAs were subjected to real-time quantitative PCR for the estimation of relative mRNA levels as described in Materials and Methods. Each point with bars shows mean $\pm$ SEM ($n=5$). * $p<0.05$, ** $p<0.01$, ET-1-treated vs. untreated cells.

buffered saline (HBS: 20 mM HEPES pH 7.4 and 100 mM NaCl) containing 16 μ g human holo-transferrin (Sigma), incubated for 20 min at room temperature, mixed with 100 μ l HBS containing various amounts of DNA mixtures and further incubated for 15 min. Fifteen microliters of DNA-liposome-transferrin complex was then overlaid on cells that had been covered with 15 μ l of serum-free DMEM and incubated for 2 h. DNA-liposome complex was confirmed to form multilamellar vesicles known to exert high transfection efficiency (22). Forty-eight hours after transfection, firefly and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) in a single-tube assay format using MicroLumat^{plus} (EG&G Berthold). The firefly luciferase activity of each sample was normalized to an internal reference standard of renilla luciferase activity. All transfections were repeated two to four times in octet, with at least three different plasmid preparations, and data from representative experiments are shown.

Flow Cytometry

VSMCs were detached with 0.25% trypsin/EDTA

(ethylenediaminetetraacetic acid), and cell suspensions were incubated for 30–60 min on ice with 1:100 mouse anti-human integrin $\alpha_v\beta_5$ monoclonal antibody (Chemicon International, Temecula, USA). The cells were then washed twice with RPMI/5% FCS and fixed with 1% paraformaldehyde. An isotype-matched, fluorescence-labeled non-binding antibody was included as a control. Subsequently the cells were stained with 0.5 mg/ml goat anti-mouse monoclonal antibody conjugated with Texas Red (Molecular Probes, OR). Fluorescence was then analyzed using a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA) to detect fluorescence, forward scatter, and size.

Cell Adhesion

Flat-bottomed 96-well microtiter plates were coated for 1 h with either 1 μ g/ml each of rat vitronectin (Sigma Chemical, St. Louis, MO) or rat fibronectin (Sigma Chemical), and then incubated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature for 30 min. The wells were rinsed twice with 0.2% BSA in serum-free DMEM (adhesion medium) prior to use. Subconfluent VSMCs were trypsinized and resuspended (10^6 cells/ml) in adhesion medium. Cells (5×10^4 cells/well) were seeded on wells precoated with or without vitronectin or fibronectin and incubated at 37°C in 5% CO₂ atmosphere for adhesion for 30 min. For the assessment of cell adhesion, cells were preincubated with 10^{-7} M ET-1 for 24 h prior to trypsinization. Suspended cells were stained with 0.05% 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM, Molecular Probes)-black acetate buffer for 30 min at room temperature. After washing, absorbance was measured at 630 nm in a Cytofluor Series 4000 fluorescence plate reader (PerSeptive Biosystems, Framingham, USA). Plates were scanned before and after washing for adherent cells. Adherence was expressed as a percentage of the fluorescence of coated wells subtracted by non-coated wells that serve as nonspecific control. Data were expressed as the mean of octet wells.

Results

Quantification of rat integrin α_v mRNA transcripts using a real-time quantitative PCR method revealed that ET-1 (10^{-7} M) time-dependently (6–16 h) decreased the steady-state integrin α_v mRNA levels in rat VSMCs, and the inhibitory effect was dose dependent (10^{-10} – 10^{-6} M) (Fig. 1). Down-regulation of integrin α_v mRNA by ET-1 (10^{-7} M) was antagonized by pretreatment with a specific ET_A receptor antagonist, BQ123 (10^{-6} M), but not with a specific ET_B receptor antagonist, BQ788 (10^{-6} M) (Fig. 1B) or any of the following compounds: the mitogen-activated protein (MAP) kinase kinase inhibitor

Fig. 2. Effect of ET-1 on stability of rat integrin α_v mRNA and protein synthesis. Left panel: Cells were incubated with (●) or without (□) ET-1 (10^{-6} M) before addition of actinomycin D (5 μ g/ml) and harvested for the indicated times; rat integrin α_v mRNA was quantified by real-time quantitative PCR. Each point represents the mean $\pm$ SEM ($n=3$); SEMs were within each point. Right panel: Cells were extracted 16 h after incubation without (control) or with cycloheximide (5 μ g/ml, CHX) or CHX plus ET-1 (10^{-6} M). Each column with a bar represents the mean $\pm$ SEM ($n=3$).

PD98059 (10^{-5} M); the PI₃ kinase inhibitors wortmannin (10^{-6} M) and LY294002 (2×10^{-6} M); the tyrosine kinase inhibitor ST638 (10^{-4} M); the Ca²⁺ channel blockers nicardipine (10^{-6} M) and benidipine (10^{-6} M); the protein kinase C inhibitor GF109203X (10^{-8} M), or the phospholipase C inhibitor U73122 (10^{-5} M) (data not shown).

We quantified integrin α_v mRNA levels after the addition of an RNA synthesis inhibitor, actinomycin D, and a protein synthesis inhibitor, cycloheximide. The integrin α_v mRNA has a short life span, with an apparent half-life of about 70 min in the presence and absence of ET-1 (Fig. 2, left panel), suggesting that suppression of integrin α_v mRNA levels by ET-1 is not due to enhanced degradation of integrin α_v mRNA. Cycloheximide added alone markedly decreased steady-state integrin α_v mRNA levels, and no further decrease in integrin α_v mRNA level was noted in the presence of cycloheximide and ET-1 (Fig. 2, right panel). These results indicate that ET-1 reduces integrin α_v mRNA transcripts via the ET_A receptor in VSMCs and suggest that the inhibitory effect on integrin α_v mRNA by ET-1 may require *de novo* protein synthesis.

Since ET-1 suppressed the constitutive expression of integrin α_v , we examined whether ET-1 reduces the transcriptional activation of integrin α_v gene using the integrin α_v promoter-luciferase transcription assay. The addition of ET-1 to VSMCs reduced the reporter activity in a

Fig. 3. Effects of ET-1 on rat integrin α_v promoter activity. Cells transiently cotransfected with the integrin α_v promoter-luciferase construct and pRL-TK vector expressing renilla luciferase as an internal control were incubated with the indicated concentrations of ET-1 for 16 h or with ET-1 (10^{-7} M) in combination with BQ123 (10^{-6} M) or BQ788 (10^{-6} M). Firefly luciferase activities were normalized with renilla luciferase activity. Each point with bars shows the mean $\pm$ SEM ($n=6$). * $p < 0.005$ and ** $p < 0.01$ vs. control.

Fig. 4. Flow cytometric analysis of the cell surface expression of integrin $\alpha_v\beta_5$. A total of 10,000 cells were analyzed for each treatment with ET-1 (10^{-6} – 10^{-8} M). Relative cell numbers (vertical) and fluorescence intensity on the log scale (horizontal) are indicated.

dose-dependent fashion (10^{-9} – 10^{-6} M), and this effect was blocked by pretreatment with BQ123 (10^{-6} M) but not with BQ788 (10^{-6} M) (Fig. 3). The addition of an ETB receptor agonist, BQ3020 (10^{-6} M), had no effect (data not shown). These data demonstrate that ET-1 down-regulates rat integrin α_v gene by inhibiting transcription via the ET_A receptor.

We next studied whether ET-1 reduces the expression of integrin α_v molecules on the surface of VSMCs. Flow

Fig. 5. Effects of ET-1 on VSMC adhesion to vitronectin. Suspended VSMCs pretreated with ET-1 (10^{-6} M) were seeded and incubated for 30 min on culture wells coated with vitronectin or fibronectin. Bars represent the mean $\pm$ SEM of bound VSMCs relative to total cells. * $p < 0.01$ vs. control cells.

cytometry analyses using fluorescence-labeled monoclonal antibody to rat integrin $\alpha_v\beta_5$ revealed that the expression of integrin $\alpha_v\beta_5$ on the cell surface was reduced after incubation with ET-1 in a dose-dependent fashion (10^{-8} – 10^{-6} M) (Fig. 4). Taken together, these data provide evidence that ET-1 reduces expression of integrin $\alpha_v\beta_5$ on the cell surface of VSMCs.

Finally, we performed an adhesion assay using VSMCs seeded on plastic dishes precoated with vitronectin or fibronectin or not precoated. ET-1 (10^{-7} M) inhibited the adhesion of VSMCs to vitronectin but not to fibronectin (Fig. 5). These results suggest that ET-1 preferentially prevents VSMCs from vitronectin adhesion *via* inhibition of integrin α_v expression.

Discussion

The extracellular matrix is an important factor for regulating VSMC adhesion and spreading. Cellular integrin receptors represent the linkage between the cytoskeleton, the extracellular matrix, and the intracellular signaling apparatus. Accumulating lines of evidence suggest that integrins play an important role in cardiovascular remodeling (7). Integrins that bind to the arginine-glycine-aspartic acid motif, such as integrin $\alpha_v\beta_3$, have been shown to inhibit angiogenesis, leading to inhibitions of tumor growth and restenosis after vascular injury. Integrin binding initiates signals that stimulate the tyrosine phosphorylation of a number of cellular proteins, such as ras, raf-1, focal adhesion kinase, and members of the MAP kinase family, which have been shown to regulate a variety of functions, including cell cycle transition, mitogenesis, apoptosis, cell adhesion, and motility (1, 7, 23). Although the expression of integrins has been shown to be influenced by several

cytokines and growth factors (24, 25), there is no previous report addressing the interaction between ET-1 and integrins.

In the present study, we examined the effects of ET-1 on integrin α_v expression in rat VSMCs in four experimental settings: 1) quantification of endogenous integrin α_v gene copies using real-time quantitative PCR; 2) examination of the promoter activity of integrin α_v gene after transient transfections of reporter constructs; 3) detection of cell surface integrin $\alpha_v\beta_5$ molecules using flow cytometry analysis; and 4) measurement of vitronectin adhesion. We obtained complementary results in each assay, showing that the integrin α_v gene is down-regulated by ET-1. Using the ABI Prism 7700 Sequence Detection System to quantitate rat integrin α_v mRNA transcripts, we have clearly shown that ET-1 time- and dose-dependently down-regulated integrin α_v gene expression in rat VSMCs. This effect was mediated *via* the ET_A receptor in VSMCs, since a specific ET_A receptor antagonist, but not an ET_B receptor antagonist, abrogated the inhibitory effect of ET-1. Furthermore, the present study revealed that reduction of integrin α_v expression by ET-1 led to decreased integrin α_v molecules on the cell surface and subsequent inhibition of adhesion by vitronectin, whose receptors consists of integrin α_v .

The exact intracellular signaling pathway(s) downstream of the ET_A receptor remains an open question. ET-1 has been shown to cause increased intracellular free Ca^{2+} concentrations (11) and to induce MAP kinase (13, 14), PI₃ kinase (26), and protein kinase C (27) in a variety of cells. However, the inhibitory effect on integrin α_v expression by ET-1 was not abrogated by any compounds known to selectively inhibit such pathways in this study. On the other hand, the results obtained with a protein

synthesis inhibitor, cycloheximide, suggest that the effect by ET-1 may require *de novo* protein synthesis.

Changes in integrin α_v gene expression are most likely due to transcriptional regulation, since the activity of integrin α_v promoter-luciferase construct was similarly reduced by ET-1, and integrin α_v mRNA degradation was not affected by ET-1. Both mouse and human integrin α_v promoters contain many common consensus sequences, such as those for Sp1, Ets, AP-2, and GATA-1, but lack both TATA or CCAAT boxes (28). In contrast to human integrin α_v promoter, in which a region spanning from -147 to +170 directs its transcriptional activity, mouse genes are under the control of a different region (positions -108 to +22). The 5'-flanking region of the mouse integrin α_v gene we cloned in the present study contains not only sequences necessary for its transcriptional activity but also regions required for ET-1-induced suppression. The exact responsible sites downstream from ET-1 stimulation remain unknown, since previous studies have not revealed any elements involved in negative regulation. TATA-less promoters, including integrin α_v , have a consensus sequence of an initiator element near each transcription initiation site critical for positioning RNA polymerase II (29). The initiator element, on the other hand, is also under the control of the potent transcription inhibitory effect of an immediate early response gene, c-Myc (30). Since ET-1 potently stimulates the expression of c-Myc in VSMCs (31) and ET-1-induced inhibition of integrin α_v expression involves the cycloheximide-sensitive pathway, the inhibitory effect of ET-1 on integrin α_v transcription may be mediated *via* a c-Myc-dependent mechanism that requires *de novo* protein synthesis. The characterization of the putative *cis*-elements and the transcription factors responsible for the ET-1-induced inhibition of integrin α_v gene transcription needs to be determined.

ET-1 was found to have diverse actions on VSMCs, including vasoconstriction, mitogenesis, migration, and apoptosis survival, which are mediated mainly *via* the ET_A receptors (13, 32). The present study adds the novel function of ET-1 as a regulator of vascular integrin to the list of its diverse actions. It has been shown that ET-1 is overexpressed in atherosclerotic plaques (33), that migration of human VSMCs by lysophosphatidylcholine is enhanced by ET-1 (16), and that vitronectin stimulates VSMC migration *via* its receptor activation (3-5). Therefore, it is possible to speculate that the potent pro-migratory and proliferative effect by ET-1 may be counteracted by the decreased cell surface expression of integrin α_v and vitronectin binding to VSMCs. The inhibitory functions of ET-1 on vascular integrin α_v expression may serve as an inherent feedback mechanism to prevent overshooting of ET-1 actions in the process of vascular remodeling.

In conclusion, we have demonstrated a novel function of ET-1 as a negative regulator of integrin α_v expression.

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