Molecular Mechanism of Fibronectin Gene Activation by Cyclic Stretch in Vascular Smooth Muscle Cells*

Fibronectin plays an important role in vascular remodeling. A functional interaction between mechanical stimuli and locally produced vasoactive agents is suggested to be crucial for vascular remodeling. We examined the effect of mechanical stretch on fibronectin gene expression in vascular smooth muscle cells and the role of vascular angiotensin II in the regulation of the fibronectin gene in response to stretch. Cyclic stretch induced an increase in vascular fibronectin mRNA levels that was inhibited by actinomycin D and CV11974, an angiotensin II type 1 receptor antagonist; cycloheximide and PD123319, an angiotensin II type 2 receptor antagonist, did not affect the induction. In transfection experiments, fibronectin promoter activity was stimulated by stretch and inhibited by CV11974 but not by PD123319. DNA-protein binding experiments revealed that cyclic stretch enhanced nuclear binding to the AP-1 site, which was partially supershifted by antibody to c-Jun. Site-directed mutation of the AP-1 site significantly decreased the cyclic stretch-mediated activation of fibronectin promoter. Furthermore, antisense c-jun oligonucleotides decreased the stretch-induced stimulation of the fibronectin promoter activity and the mRNA expression. These results suggest that cyclic stretch stimulates vascular fibronectin gene expression mainly via the activation of AP-1 through the angiotensin II type 1 receptor.

Extracellular matrix of the vascular wall plays an important role in pathophysiological changes including vascular remodeling and atherosclerosis in response to hypertension. Fibronectin (FN) is an important component of the extracellular matrix and is implicated functionally in the regulation of several cellular processes, including cell adhesion, migration, transformation, and motility and wound healing. FN has been found to modulate the phenotype of vascular smooth muscle cells (VSMCs) and regulate VSMC growth (1). We previously found that angiotensin II (Ang II) enhances transcription of the FN gene through the Ang II type 1 receptor (AT1 receptor) in VSMCs, at least in part via activation of the rat FN promoter AP-1 binding motif (rFN/AP-1) (2). Although rFN/AP-1 may not be involved in the regulation of FN gene expression in cells other than VSMCs (3, 4), this result proposes that rFN/AP-1 is functionally important for the regulation of vascular FN expression in response to various stimuli. Accumulated evidence suggests that hemodynamic forces (including stretch and shear stress) as well as endocrine factors (such as Ang II) are among the most important factors implicated in the physiology and pathophysiology of the vascular wall in vivo. Interactions between extracellular matrix proteins and cellular receptors can transduce signals that lead to changes in shape, motility, and growth of VSMCs. Thus, investigation of mechanical stress-mediated regulation of the extracellular matrix and the tissue renin-angiotensin system in VSMCs may be important for the elucidation of a molecular mechanism of vascular remodeling and atherosclerosis. With respect to vascular FN regulation, several in vivo studies have reported that hypertension activates the vascular renin-angiotensin system and induces expression of vascular FN (5, 6). Actually, we have recently examined expression of the tissue renin-angiotensin system and FN genes in inbred Dahl Iwai salt-sensitive (DS) and salt-resistant (DR) rats (7). The expression of tissue angiotensinogen, AT1 receptor, and FN is regulated differently in DS and DR rats, and salt-mediated hypertension in DS rats stimulates the aortic FN gene, with activation of the tissue renin-angiotensin system in a tissue-specific manner.

Although our previous studies and others suggest that Ang II in vitro and hypertension in vivo increase vascular FN expression, the molecular mechanisms of mechanical stress-mediated regulation of vascular FN is still unclear. Therefore, in the present study, we examined the effects of cyclic stretch on gene expression of extracellular matrix components (FN and collagens) and renin-angiotensin system components (angiotensinogen, angiotensin-converting enzyme [ACE], and AT1 receptor) in VSMCs. Cyclic stretch of VSMCs induced mRNA expression of FN, collagen, ACE, and AT1 receptor, and Ang II secreted in

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* This work was supported by grants from the Ministry of Education, Science, and Culture of Japan, Uehara Memorial Foundation, Ichiro Kanehara Foundation, and Yokohama Foundation for Advancement of Medical Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: FN, fibronectin; VSMCs, vascular smooth muscle cells; Ang II, angiotensin II; AT1 receptor, angiotensin II type 1 receptor; rFN/AP-1, rat fibronectin promoter AP-1 binding motif; DS rat, Dahl Iwai salt-sensitive rat; DR rat, Dahl Iwai salt-resistant rat; ACE, angiotensin-converting enzyme; AMD, actinomycin D; AT2 receptor, angiotensin II type 2 receptor; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; TK, thymidine kinase.

This paper is available on line at http://www.jbc.org

Vol. 275, No. 44, Issue of November 3, pp. 34619–34627, 2000
Printed in U.S.A.
an autocrine/paracrine manner is involved in stretch-induced expression of FN. Furthermore, a specific promoter region, rFN/AP-1, may play an important role in the stretch-mediated increase in FN gene transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 medium, fetal calf serum, penicillin, and streptomycin were obtained from Life Technologies, Inc. Ang II, actinomycin D (AMG), cycloheximide, and sarasalin were purchased from Sigma. AT1 receptor-specific antagonist CIV11974 and Ang II type 2 receptor (AT2 receptor)-specific antagonist FD123319 were supplied by TaqPCR Chemicals and Parke Davis, respectively. 

**Cell Culture and Application of Cyclic Stretch to Cultured Cells**—VSMCs were isolated and cultured as described previously (2). For the experiments, cells at approximately 80% confluence in dishes were made quiescent by incubation with serum-free RPMI 1640 medium for 24 h, unless otherwise stated.

Cells grown in 6-well silicone elastomer-bottomed culture plates (Flexcell Corp.) were subjected to cyclic mechanical stretch in a FX-3000 Flexcell Strain Unit with BioFlex Loading Stations (Flexcell Corp.). The strain unit, a modification of the device described by Banes et al. (8) and Gilbert et al. (9), consists of a computer-controlled vacuum unit and a base plate to hold the culture dishes on the Loading Stations. Vacuum is sequentially applied, 1 Hz, 0.5 s on-time at 80 cycles per min to the rubber-covered dishes via the base plate, which is placed in a standard CO2 tissue culture incubator. When vacuum is applied to a flexible culture dish with the Flexcell Strain Unit and Loading Stations, the bottom deforms across the post face, creating uniform radial and circumferential strain to the bottom of culture dish, principally as described previously (10). This process allows cell culture in a uniform mechanically active environment so that all cells stretched over the loading station surface receive the same amount of strain. Previous studies showed that cyclic stretch of VSMCs with the Flexcell Strain Unit induced increases in DNA synthesis and total protein synthesis, thereby suggesting that this system is useful for in vitro analysis of the processes involved in vascular remodeling (11, 12).

To determine possible cellular injury due to mechanical stretch, viability of stretched VSMCs was constantly monitored by either the trypsin blue dye exclusion test or assay of the mitochondrial reduction of trypan blue dye or assay of the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (Chemicon International Inc.) (13). The attached cells showed a viability of 82 ± 7, 84 ± 7, and 87 ± 6% in the 30% stretched, 20% stretched, and nonstretched regimens (12 h), respectively. The viability of stretched cells did not differ from that of nonstretched VSMCs throughout the experiments in this study.

**RNA Isolation and Northern Blot Hybridization**—Northern blot analysis was performed essentially as described previously (2). Twenty micrograms of each total RNA sample were denatured, electrophoresed, and transferred to a nylon membrane. Hybridization proceeded in the presence of 70% ethanol, as described previously (15). Corresponding cDNA fragments for the AP-1 element; 5'-GCAG- GATTtttttGGGAGTCGGGA-3', −111 to −88, and mutated nucleotides are shown in bold) were also synthesized and purified.

**Plasmid Construction, DNA Transfection, and Chloramphenicol Acetyltransferase (CAT) Assay**—Chloramphenicol acetyltransferase (CAT) activity to correct for differences in transfection efficiency. The conversion ratios of [14C]chloramphenicol were measured with BSA2000.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**—VSMCs were pretreated with Ang II receptor antagonists or signaling inhibitors for 30 min and subjected to 6 h of cyclic stretch (20%). Nuclear extracts from VSMCs were prepared with a modification of the protocol of Dignam et al. (18) and Swick et al. (19). EMSA was performed essentially as described previously (2). For the supershift experiments, antibodies with epitopes specific to c-Jun, JunB, and conserved regions of the Fos family (Santa Cruz Biotechnol-
was observed between 5 and 20% stretch (Fig. 2A). Maximum activation of FN mRNA was obtained by 20% cyclic stretch, and the levels of FN mRNA stimulated by 25 and 30% cyclic stretch were almost the same as that stimulated by 20% stretch. Therefore, subsequent experiments were performed by 20% cyclic stretch of VSMCs in serum-free medium.

Previous studies showed that specific extracellular matrix proteins modulated the mechanical strain-induced responses of VSMCs (11, 21). To examine a possible effect of extracellular matrix proteins on the induction of FN mRNA by cyclic stretch in VSMCs, we coated the silicone elastomeric bottom of dishes with type I collagen, elastin, FN, or laminin (Type I collagen was used as a control, as stated earlier). However, there were no significant differences in the degree of activation of FN mRNA expression induced by 20% cyclic stretch among these extracellular matrix proteins (Fig. 2B).

Effects of Transcriptional Inhibitor and Protein Synthesis Inhibitor on Cyclic Stretch-mediated Increase in FN mRNA—To determine whether de novo RNA or protein synthesis was required for a cyclic stretch-induced increase in FN mRNA, VSMCs were pretreated with AMD or cycloheximide for 30 min and stretched (20%) for 6 h. The RNA synthesis inhibitor AMD almost abolished the cyclic stretch-mediated increase in FN mRNA, whereas the induction of FN mRNA by cyclic stretch was not significantly altered by cycloheximide (Fig. 3A). None of these inhibitors alone had any influence on the expression of FN mRNA in control VSMCs. These results indicate that de novo mRNA transcription is required for the induction of FN mRNA expression by cyclic stretch, but that the effects of cyclic stretch do not require de novo protein synthesis to stimulate the expression of FN mRNA.

Putative Role of Ang II Receptor in Cyclic Stretch-mediated
For 6 h. Treatment of VSMCs with the AT1 receptor-specific antagonist CV11974 (10 μM) significantly decreased the stimulatory effect of cyclic stretch (Fig. 3B). In contrast, incubation of cells with the AT2 receptor-specific antagonist PD123319 (100 μM) did not affect the response to cyclic stretch at all. None of these Ang II receptor antagonists alone had any influence on the expression of FN mRNA in control VSMCs. It is possible that the lack of inhibition by PD123319 was due to an insufficient dose of the inhibitor. However, preincubation of VSMCs with increasing doses of PD123319 (100 and 500 μM) did not affect the response to cyclic stretch at all (data not shown). These results indicate that cyclic stretch activates FN mRNA expression, at least partly, through an AT1 receptor-dependent pathway in VSMCs.

Effects of Cyclic Stretch on Ang II Secretion into Culture Medium—A previous study using the Flexercell strain system showed that concentration of immunoreactive Ang II in the conditioned medium was elevated by cyclic stretch of VSMCs (12), and in the present study cyclic stretch of VSMCs caused increases in ACE and AT1 receptor mRNA expression. To determine whether the autocrine/paracrine-secreted Ang II actually contributes to the increase in FN mRNA expression in response to cyclic stretch, incubation medium obtained from VSMCs that were stretched for 12 h was transferred to VSMCs cultured on regular culture dishes. The addition of conditioned media for 12 h significantly enhanced FN mRNA levels in recipient VSMCs (Fig. 4A). Pretreatment of the VSMCs with the AT1/AT2 receptor antagonist saralasin or CV11974 completely blocked the increase induced by the addition of conditioned media, whereas pretreatment with PD123319 or BQ123, an endothelin-1 type A receptor antagonist, did not affect the increase in FN mRNA levels. Furthermore, the culture medium from stretched VSMCs showed a significant increase in immunoreactive Ang II compared with that from nonstretched control VSMCs (Fig. 4B). Thus, Ang II secreted from stretched VSMCs is suggested to be the major molecule in conditioned medium that up-regulates FN mRNA levels.

Cyclic Stretch-mediated Activation of FN Promoter—We examined whether cyclic stretch activates transcription directed by the FN promoter. We transfected a rat FN promoter (−1908 to +136 of the transcriptional start site)-CAT chimeric gene (rFN-CAT) into VSMCs and stimulated VSMCs with cyclic stretch (20%) for 6 h. Cyclic stretch increased CAT activity of rFN-CAT by 4.1-fold (Fig. 5). We also examined what type of Ang II receptor(s) was involved in mediating the enhanced CAT activity through the FN promoter in response to cyclic stretch. Incubation of VSMCs with 10 μM saralasin or CV11974 significantly decreased the stimulatory effect of cyclic stretch, whereas incubation of cells with 100 μM PD123319 did not affect the response to cyclic stretch at all. Thus, the AT1 receptor appears to play a role in the cyclic stretch-mediated increase in FN promoter activity in VSMCs.

Cyclic Stretch-mediated Increase in AP-1 Binding Activity—The results of a DNA transfection study showed that cyclic stretch stimulated transcription directed by the FN promoter in VSMCs. Previous studies showed that the 5′-flanking region of the rat FN gene contained the motifs for transcription factors E4TF1, AP-1, AP-2, PEA2, Sp1, and CRE (24). Among them, the AP-1 binding motif is known to be one of the targets of Ang II-mediated transcriptional activation (22), and we have recently shown that a rat FN promoter region from −473 to −447 of the transcriptional start site (rFNAP-1), which contains an AP-1 binding motif, is involved in the Ang II-mediated transcriptional activation of the FN gene (2). To examine the effects of cyclic stretch on binding of nuclear factors to this element, we first performed DNase I footprint analysis. VSMCs were
subjected to cyclic stretch (20%) for 6 h. The results showed that a sequence from −458 to −439 in the rFN/AP-1 element was protected from digestion by DNase I (lanes 2 and 3, denoted by the hatched box, Fig. 6A). The pattern of DNase I footprinting disclosed increased protection upon 20% cyclic stretch (1) (Fig. 6A, lanes 4 and 5). In footprinting competition assay, this protection was inhibited by the addition of nonlabeled rFN/AP-1 element to the reaction mixture (Fig. 6A, lane 6).

We next carried out EMSA using rFN/AP-1 as a probe. VSMCs were subjected to cyclic stretch (20%) for 6 h. Incorporation of VSMC-nuclear extracts with the 32P-labeled rFN/AP-1 produced a single shifted band, and cyclic stretch significantly increased the intensity of this band (Fig. 6B, lanes 1 and 2). In electrophoretic mobility shift competition assay, the shifted band was specifically competed out by the unlabeled rFN/AP-1 element, but not by the rFN/m[AP-1] element which contained substitution mutations interrupting the AP-1 binding motif (Fig. 6B, lanes 3–6).

Incubation of VSMCs with 10 μM saralasin or CV11974 significantly decreased the stretch-induced nuclear binding to rFN/AP-1, whereas incubation of cells with 100 μM PD123319 did not affect the nuclear binding (Fig. 7, lanes 3–8). Thus, AT1 receptor, but not AT2 receptor, is suggested to be involved in the cyclic stretch-mediated increase in rFN/AP-1 binding activity in VSMCs.

**Functional Importance of rFN/AP-1 Element in Stretch-mediated Activation**—From the above results, the rFN/AP-1 element seems to exert a major influence on cyclic stretch-mediated transcriptional activity of the FN gene in VSMCs. Thus, to evaluate the functional significance of the rFN/AP-1 element in stretch-mediated FN promoter activity, we first fused the rFN/AP-1 or rFN/m[AP-1] element in 5′ to 3′ orientation upstream of a herpes simplex virus-thymidine kinase (TK) promoter-CAT hybrid gene. As shown in Fig. 8A, rFN/AP-1/TK-CAT elicited stretch-induced expression of the CAT-reporter gene (3.6-fold activation). On the other hand, the rFN/m[AP-1] element (rFN/m[AP-1]/TK-CAT) did not confer the stretch-mediated activation of CAT expression.

To further establish the functional roles of the rFN/AP-1 element in directing cyclic stretch-induced CAT expression in the native FN promoter context, we assayed effects of a mutation that disrupted binding of nuclear factors to this element. The FN promoter (−1908 to +136) linked upstream of the CAT coding gene. Forty-eight hours after transfection, VSMCs were pretreated for 30 min with Ang II receptor antagonists, followed by cyclic stretch (20%) for 6 h, and promoter activity was estimated by CAT assay. CAT activities were measured with an Imaging Analyzer BAS2000 and expressed relative to those achieved with cell extracts from control VSMCs (the mean CAT activity of control VSMCs is expressed as 100%). Bars represent mean ± S.E. of four independent transfection experiments. *, p < .05 versus stretch (+) without antagonist.
motif on rFN/AP-1 binding activity of FN promoter.

respectively. indicate specific DNA-protein complex and free probe, respectively. of the competitor DNA was added to the reaction mixture. The probe (rFN/AP-1). In competition assay, 10- or 100-fold molar excess of the nonlabeled rFN/AP-1 element was added to the reaction mixture.

Nuclear extracts from VSMCs (20%) for 6 h, and rFN/AP-1 binding activity was estimated by EMSA. In competition assay, 100-fold molar excess of the nonlabeled rFN/AP-1 element was added to the reaction mixture.

LANE 1 contained no nuclear extract. The rFN/AP-1 element and protected region on footprinting are denoted by the open and hatched boxes, respectively. AP-1 binding motif is underlined. B, analysis of nuclear factor binding to the FN/AP-1 element and effects of cyclic stretch by EMSA. VSMCs were stimulated with cyclic stretch (20%) for 6 h, and rFN/AP-1 binding activity was estimated by DNase I footprint analysis. In competition assay, 100-fold molar excess of the nonlabeled rFN/AP-1 element was added to the reaction mixture. Lane 1 contained no nuclear extract. The rFN/AP-1 element and protected region on footprinting are denoted by the open and hatched boxes, respectively. AP-1 binding motif is underlined. B, analysis of nuclear factor binding to the FN/AP-1 element and effects of cyclic stretch by EMSA. VSMCs were stimulated with cyclic stretch (20%) for 6 h, and rFN/AP-1 binding activity was estimated by DNase I footprint analysis.

AP-1 family of transcription factors, a supershift assay was performed. An antibody recognizing conserved region epitopes of the Fos family and antibodies recognizing specific region epitopes of c-Jun or JunB were used. The protein-DNA complex formed by the rFN/AP-1 element probe was not supershifted by the antibodies to either Fos or JunB, whereas the shifted complex was partially supershifted in the presence of the antibody to c-Jun antibody (Fig. 9A). These results indicate that the c-Jun protein is one of the components of the protein-DNA complex, which is formed by the binding of nuclear factors to rFN/AP-1 in response to cyclic stretch of VSMCs.

To examine whether c-Jun was actually involved in the cyclic stretch-mediated transcriptional regulation of FN gene, stretched VSMCs were exposed to antisense oligodeoxynucleotides complementary to the c-jun mRNA translation initiation sites, thereby inhibiting c-Jun protein synthesis. This approach has previously been shown to be successful in inhibiting c-Jun protein synthesis in cultured cells (15). VSMCs were transfected by rFN-CAT and subjected to cyclic stretch (20%) for 9 h in the presence of sense or antisense c-jun oligodeoxynucleotides. The addition of 30 μM antisense c-fos oligodeoxynucleotides or sense c-jun oligodeoxynucleotides, which were used as controls, did not affect the stretch-induced increase in CAT expression, whereas the presence of 30 μM antisense c-jun oligodeoxynucleotides significantly inhibited the induction of CAT activity directed by rFN-CAT (Fig. 9B). Furthermore, antisense c-jun oligodeoxynucleotides also inhibited stretch-mediated increase in FN mRNA expression, while antisense c-fos oligodeoxynucleotides or sense c-jun oligodeoxynucleotides did not affect the induction (Fig. 9C). These antisense experiments indicate that c-Jun is functionally involved in cyclic stretch-induced activation of the FN gene in VSMCs.

**DISCUSSION**

Previous results suggest that elevation of blood pressure and activation of the vascular renin-angiotensin system may be synergistically perceived by vascular cells as a signal that is transduced to increase the expression of aortic FN (21). Ang II stimulates VSMC growth and enhances the production of FN and collagen in VSMCs, mesangial cells, and renal fibroblasts (23–25). In addition, a recent study examined the effects of AT1 receptor blockade on the gene expression of immediate-early response genes, including c-jun and c-fos, and FN after endothelial denudation of the carotid artery by balloon catheter in Harlan Sprague-Dawley rats (26). The results showed that blockade of the AT1 receptor inhibited the induction of AP-1 and FN in an injured rat artery.

In this study, we showed that cyclic stretch of VSMCs enhanced the mRNA expression of ACE and AT1 receptor as well as FN and demonstrated that an AT1 receptor antagonist but not an AT2 receptor antagonist inhibited the cyclic stretch-induced expression of the FN gene. Furthermore, we have found that an AP-1-like element of the FN promoter plays a role in the stretch-mediated activation of the vascular FN gene. We have recently shown that Ang II activates transcription of the FN gene through the AT1 receptor by activation of AP-1 in...
FIG. 8. Effects of a mutation of rFN/AP-1 on promoter activity. A, stretch-mediated transcriptional activation directed by rFN/AP-1 linked upstream of a heterologous TK promoter. VSMCs were transfected with a plasmid rFN/AP-1/TK-CAT (5 μg) or rFN/m[AP-1]/TK-CAT (5 μg). Forty-eight hours after transfection, VSMCs were subjected to cyclic stretch (20%) for 6 h, and promoter activity was estimated by CAT assay. Bars represent mean ± S.E. of four independent transfection experiments. *, p < .05 versus stretch (+) with rFN/AP-1/TK-CAT. Arrowheads indicate different forms of acetylated [14C]chloramphenicol which represent promoter activity. B, effects of mutation of cis-elements in the native FN promoter on stretch-induced transcriptional activation. VSMCs were transfected with a plasmid rFN-CAT (5 μg), rFN/m[AP-1]-CAT (5 μg), rFN/m[Sp1]-CAT (5 μg), rFN/m[CRE]-CAT (5 μg), CAT assay was performed as described in A. Bars represent mean ± S.E. of four independent transfection experiments. *, p < .05 versus stretch (+) with rFN-CAT.
Bars in the legend of Fig. 5.

The presence of sense or antisense c-fos (c-fos S), or antisense c-jun (c-jun AS), and FN mRNA/18 s ribosomal RNA (IJS) levels were estimated by Northern blot analysis (total RNA 20 μg). The levels of FN mRNA expression were measured as described in the legend of Fig. 1. Bars represent mean ± S.E. of four independent experiments. *, p < .05 versus stretch (+) without oligodeoxynucleotides.
Cyclic Stretch and Vascular Fibronectin

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