Hypertension Induces Alternatively Spliced Forms of Fibronectin in Rat Aorta

Izumi Takasaki, Aram V. Chobanian, Wilfred S. Mamuya, and Peter Brecher

Fibronectin expression was shown recently to increase in the rat aorta in response to experimental hypertension. Fibronectin is known to alter the phenotype of vascular smooth muscle and endothelial cells, and relative changes in the expression of different isoforms of fibronectin, generated by alternative splicing and distinguished by the absence or presence of inserts designated as EIIIA, EIIIB, and V, may reflect a change in cell phenotype. In the present study we examined the expression of alternatively spliced forms of aortic fibronectin during deoxycorticosterone-salt hypertension. Aortic RNA was analyzed quantitatively using Northern blot analysis and ribonuclease protection assays. Using Northern blot analysis, deoxycorticosterone-salt treatment for 21 days led to a 4.9-fold increase in EIIIA fibronectin messenger RNA, while EIIIB and V forms increased by 2.6- and 2.5-fold, respectively. As determined by ribonuclease protection assays, the percentage of fibronectin transcripts containing either EIIIA, EIIIB, or V in control aorta was 7.3%, 19%, and 40%, respectively. The percentage of EIIIA transcripts increased 42% over control levels after 21 days of deoxycorticosterone-salt treatment, whereas no proportionate change in the other alternatively spliced forms was found. Thus, all forms increased, but a selective increase in the EIIIA form was induced. Analogous increases in each of the fibronectin isoforms were found in the spontaneously hypertensive rats when compared with age-matched Wistar-Kyoto or Wistar rats, and 40-week-old animals showed increases over 10-week-old animals in all strains, consistent with an age-dependent increase in aortic fibronectin expression. (Hypertension 1992;20:20-25)

KEY WORDS • fibronectins • aorta • experimental hypertension • RNA splicing rat studies

Hypertension produces diverse biochemical and functional changes in vascular cells. Alterations in the aortic extracellular matrix of hypertensive animals have been documented, and it is generally accepted that changes in the composition of the extracellular matrix can influence the phenotype of both vascular smooth muscle and endothelial cells. Fibronectin is a matrix-associated glycoprotein that exists in several forms due to alternate splicing of a single gene, the different isoforms distinguished by the presence or absence of exon products, which in the rat are designated as EIIIA, EIIIB, and V. Through an interaction with specific cellular receptors known as integrins, fibronectin influences cellular functions that include motility, differentiation, and many of the events associated with wound healing. The role of fibronectin in vascular biology is incompletely understood. Although there are numerous studies where cultured endothelial and smooth muscle cells were used to establish a relation between fibronectin and cell phenotype, the presence of fibronectin in intact vascular tissue has been shown mainly by histochemical procedures. Of particular interest was a recent report showing the appearance of a specific isoform containing the EIIIA isoform in both balloon-injured intima from rat aorta and atherosclerotic plaque from human arteries. In a recent series of studies, we have shown that steady-state messenger RNA (mRNA) levels for rat aortic fibronectin increase in different forms of experimental hypertension as does the total fibronectin content, and we have developed an in vitro procedure using rabbit or rat aortic rings to examine fibronectin biosynthesis. In the present study, by examining the expression of the different alternatively spliced forms of fibronectin in hypertensive rats, we have shown that all forms of fibronectin increase and that there is a selective induction of the isoform containing the EIIIA insert.

Methods

Materials

Gene Screen Plus nylon membrane, [α-32P]dCTP, [α-32P]CTP, Ecolonfluor, and Protosol (0.5 M solution) were obtained from DuPont/New England Nuclear Corporation, Boston, Mass. A multiprime labeling kit, and RNA ladders were purchased from Ambion Inc., Austin, Tex.

Animals

Male Wistar rats, 250–300 g, were obtained from Charles River Breeding Laboratories, Wilmington,
The mixture was extracted with phenol:chloroform and 1% SDS for 15 minutes at 37°C to remove nucleases. The samples were treated with 20 \% in 10 mM Tris (pH 7.5), 300 mM NaCl, and 5 mM EDTA. Excess unhybridized probe was digested with a mixture of RNase T1 (65 units) and RNase A (0.33 units) and the RNA was precipitated with ethanol. The pellet was resuspended in RNA loading buffer, denatured at 90°C for 4 minutes, and rapidly applied to 5–8% sequencing gels. After electrophoresis, the gel was fixed, dried, and exposed to x-ray film. Each cRNA probe contained regions encompassing alternatively spliced exons and adjacent exons common to all forms of fibronectin. Thus, RNA samples produced a large and small protected fragment reflecting the presence or absence of the indicated region, and it was possible to quantitate the relative amount of those isoforms for each sample. To do so, the radioactive bands corresponding to protected fragments were excised from the dried gel while using the autoradiograph as a reference. The gel was rehydrated with 50 \mu l distilled water and incubated with 10 ml of 5\% Protosol in Econofluor (Du Pont Company, Wilmington, Del.) at 37°C for 18 hours before scintillation counting. The protected mRNA fragments that contained EI1A (280 nt) or EI1IB (350 nt) were larger than protected fragments lacking EI1A (109 nt) or EI1IB (100 nt). Quantitation was performed by correcting for the size differences, assuming that an equal extent of label incorporation occurred for all protected fragments. The total detectable fibronectin mRNA was considered the sum of the amounts of both protected fragments, and the relative amounts were calculated by dividing the amount of exon-positive moieties by the total amount of fibronectin mRNA. To detect the V isoform, the probe used lacked the V exon, but included either side of the region where the V exon would be inserted. Thus, if the V region were absent a fully protected region of 408 nt would be found. If the V region were present, two segments, 256 nt and 152 nt in length, would be found, corresponding to the protected segments on either side of the absent V exon.

**Results**

Figure 1 shows comparative data from Northern blot analysis using total aortic RNA isolated from animals treated with DOC-salt for 7 days and from age-matched uninephrectomized controls. Systolic blood pressure in the treated group averaged 137 mm Hg as compared with 128 mm Hg in the age-matched control group on the day before killing the animals, but the differences were not significant. Heart weight/body weight ratios were higher in the treated group (3.67 \times 10^{-3} versus 3.08 \times 10^{-3}), but the differences did not achieve statistical significance. On the right side of Figure 1, each lane contains 10 \mu g total aortic RNA, and the same membrane was hybridized sequentially with each of the cDNA probes indicated. Using cDNA probes specific for each of the alternatively spliced exons from the rat fibronectin gene, an increase in total fibronectin mRNA was induced as previously described. Briefly, DOC pellets (100 mg) were implanted subcutaneously into uninephrectomized rats, and saline was substituted for drinking water. Control animals were uninephrectomized but not given DOC-salt treatment. Systolic blood pressure was measured by tail-cuff plethysmography and a photoelectric cell detector.

**Tissue**

Animals were killed with an overdose of sodium pentobarbital. Aortas were removed, taking care to avoid stretching or compression of the tissue, and placed into ice-cold buffer containing (mM) NaCl 137. KCl 2.7, NaH2PO4 4.3, KH2PO4 1.4 (pH 7.4), and glucose 11. The region of the aorta extending from the arch to the diaphragm was used in all studies. Aortas were carefully cleaned of periadventitial tissue. The dissection was performed with care to avoid unnecessary damage to the vessel. The procedure took about 10 minutes per aorta.

**RNA Isolation and Analysis**

Total RNA from aortic rings was extracted using the guanidinium thiocyanate/cesium chloride centrifugation method. RNA concentration was determined by UV spectrophotometry, and 10 \mu g total RNA was separated through 0.8% agarose/1.4 M formaldehyde gel electrophoresis. Northern blotting onto nylon membranes and hybridization with the designated probes were performed as described previously. Equal amounts of RNA in each lane were confirmed by visual examination of ribosomal RNA with ethidium bromide staining. After hybridization, the nylon membranes were washed four times for 5–10 minutes at 35°C with 0.5% sodium dodecyl sulfate (SDS) and 1X, 0.5X, 0.25X, and 0.1X standard saline citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The nylon membranes were then exposed to preflashed x-ray films (Kodak X-Omat AR) between two intensifying screens for 3 hours to 5 days at -70°C. Laser densitometry, performed using a 300-A computing densitometer (Molecular Dynamics, Sunnyvale, Calif.) was used to quantitate the relative signal intensity of the bands obtained. The response was linear over the range of intensities obtained from the Northern blots, and repetitive analysis gave almost identical numerical data.

**Ribonuclease Protection Assay**

The general procedure followed was that outlined in the instructions supplied with a kit developed by Ambion, Austin, Tex. Linearized pGEM vectors containing the appropriate complementary DNA (cDNA) were used as templates to make labeled antisense RNA probes. Hybridization was performed at 45°C for 16 hours in a solution containing 80% formamide, 40 mM PIPES (pH 6.4), 400 mM sodium acetate, and 1 mM EDTA. Excess unhybridized probe was digested with a mixture of RNase T1 (65 units) and RNase A (0.33 units) in 10 mM Tris (pH 7.5), 300 mM NaCl, and 5 mM EDTA for 30 minutes at 37°C. Yeast RNA (20 \mu g) was added, and the samples were treated with 20 \mu g proteinase K and 1% SDS for 15 minutes at 37°C to remove nucleases. The mixture was extracted with phenol:chloroform:isoamyl alcohol, and the RNA was precipitated with
clearly occurred, with the relative increase in the treated animals being 1.4-, 1.8-, and 1.8-fold that of controls for EIIIA, EIIIB, and V, respectively. Data shown in the fourth row used a nonselective fibronectin probe coding for type III repeats common to all forms of fibronectin, and the relative increase was 1.7-fold. Also shown in Figure 1 are data for actin, using a cDNA probe for \( \beta \)-actin (2.2 kb) that also cross-reacts with \( \alpha \)-actin (1.8 kb). A 33% decrease was found for \( \beta \)-actin in the treated animals when densitometric analysis was performed, and the differences were significant statistically (p<0.05). Thus, the physiological changes accompanying the early stages of DOC-salt administration were sufficient to produce an increase in steady-state mRNA levels for all alternatively spliced forms of fibronectin. The data on the left side of the figure show tissue specificity for the different forms of fibronectin mRNA tested and validate the specificity of the cDNA probes used. Liver lacks mRNA for the alternatively spliced EIIIA and EIIIB regions; lung lacks only the EIIIB region, whereas aortic tissue expressed all forms.

Figure 2 summarizes data from Northern blot analysis of total RNA from control rats and those treated with DOC-salt for 21 days. Hypertension was evident at this time; the blood pressures of control and treated rats averaged 115 and 183 mm Hg, respectively. Heart weight/body weight ratios for the treated and control rats were significantly different and averaged 4.15 \( \times \) 10\(^{-3}\) and 3.01 \( \times \) 10\(^{-3}\), respectively. Each of the samples in Figure 2 represents aortic RNA obtained from two identically treated rats, and densitometric analysis was performed on the four control samples and five DOC-treated samples. Densitometric analysis showed that DOC/salt treatment for 21 days led to a 4.9-fold increase in EIIIA fibronectin mRNA, whereas EIIIB and V forms increased by 2.6- and 2.5-fold, respectively. All the increases in fibronectin mRNA were statistically significant (p<0.01). Using probes for collagen (\( \alpha2 \), type I) and tropoelastin, representing the major extra-
cellular matrix proteins of aortic tissue, a decrease in both forms of mRNA was found. The average decrease in amounts of tropoelastin and collagen mRNA after 21 days of DOC-salt treatment was 27% and 28%, respectively. Thus, there was a clear dissociation between the effects of DOC-salt treatment on fibronectin and collagen or elastin.

To define more clearly the selective induction of fibronectin isoforms, we used ribonuclease protection assays. The cRNA probes used for these assays included regions contained within the alternatively spliced exons and also contained adjacent type sequences that were common to all forms of fibronectin. Thus, it was possible to estimate the amount of fibronectin mRNA containing the exon in question relative to the amount of total fibronectin mRNA. Figure 3A summarizes data on the expression of fibronectin mRNA either lacking or containing the inserts designated EIIIA or EIIIB in aortas from control animals or those treated with DOC-salt for 21 days. There was uniformly more fibronectin in the treated animals relative to controls, consistent with Northern blot analysis. Using the procedures described in “Methods,” it was possible to quantitate the relative amount of fibronectin transcripts containing each of the alternatively spliced exons both in control and treated samples. Using four separate samples from the control group and five samples from the animals treated with DOC-salt, each sample representing RNA from two rat aortas, the percentage of fibronectin transcripts containing either EIIIA or EIIIB in control aorta was 7.3±1% and 19.3±1.2%, respectively. The corresponding values for the DOC-salt–treated samples were 10.35±0.7% and 15.5±1.3%. The selective increase in the relative amount of EIIIA transcripts was statistically significant (p<0.05), but no significant changes in EIIIB were noted. In separate experiments representative of those shown in Figure 3B, comparable values for the relative amount of fibronectin mRNA containing the V insert were obtained and in the control group averaged 39.9±1.4%, whereas in the DOC-salt–treated group the average was 42.6±1.2%. This difference was not significant. Thus, DOC-salt treatment led to a significant increase only in the relative amount of fibronectin mRNA containing the EIIIA insert.

The increases in aortic fibronectin isoforms also could be detected in a genetic hypertensive model. Figure 4 shows representative Northern blot analysis for each of the fibronectin isoforms in 10- and 40-week-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. For additional comparison, the Wistar strain also was included. In each case, there was an age-dependent increase in the fibronectin isoforms in all strains, and there clearly was more of each isoform in the hypertensive strain compared with either WKY or Wistar controls.

**Discussion**

These studies show an induction of each major isoform of fibronectin in aortic tissue associated with the hypertensive state and a selective increase in the EIIIA
forms of fibronectin were selectively expressed in vascular tissue during embryogenesis. Developing embryos have fibronectin mRNA that is mainly A*B*. Active cell proliferation and migration are occurring at this time, suggesting that these exons may be involved in either of these processes. Purified isoforms have been used in transformation assays, but the results indicate that the ability of fibronectin to revert the morphology of transformed cells does not reside in the EIIIA, EIIIB, or V regions. Experiments using those pure isoforms did suggest, however, that the inclusion of the EIIIA or EIIIB segments may enhance the ability of fibronectin to incorporate into the existing matrix.

Our studies also show that the V isoform represented between 40% and 50% of the total transcripts both in normotensive and hypertensive animals. It was not possible to determine the distribution of the V segment in molecules also containing EIIIA or EIIIB. The V segment, also known as the IIICS region or the type III connecting segment, had been implicated as capable of modulating adhesion, since it appears to contain additional sites for cell adhesion. Alternative splicing of the V region has been shown to decrease spreading and increase migration rates of bovine aortic endothelial cells. These effects were not observed with bovine aortic smooth muscle cells. The results suggest that various regions of the fibronectin molecule may be able to interact differentially with different cell populations to promote attachment and spreading and that alternative splicing of the V region may modulate this process. Further evidence for this hypothesis has been obtained from studies using pure homodimeric fibronectin, produced in heterologous mammalian cells that do not themselves produce endogenous fibronectin. These studies indicate that only the forms of fibronectin that contain the V segment will promote the spreading of a lymphoid cell line.

The functional role of alternatively spliced forms of fibronectin in the aorta during the onset of experimental hypertension has not been delineated. It seems plausible that fibronectin isoforms can facilitate both migration and proliferation of cells, as has been suggested during embryogenesis and wound healing, and cellular events that could be related to the hypertrophy, hyperplasia, and vascular remodeling associated with hypertension. Furthermore, changes in the extracellular matrix could lead to altered signaling into the cell through the interactions with integrins, perhaps facilitating a unique set of responses to growth factors that would ultimately influence the cell phenotype.

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