Molecular mechanisms of aortic wall remodeling in response to hypertension

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Objective: The molecular basis of vascular response to hypertension is largely unknown. Both cellular and extracellular components are critical. In the current study we tested the hypothesis that there is a balance between vascular cell proliferation and cell death during vessel remodeling in response to hypertension.

Methods: A midthoracic aortic coarctation was created in rats to induce an elevation of blood pressure proximal to the coarctation. The time course was 1 and 3 days and 1, 2, and 4 weeks for the study of the proximal aorta. Ribonuclease protection assay and Western blot analysis were used to evaluate gene expression of growth and apoptosis-related cytokines with two sets of multiple probes, rCK-3 and rAPO-1. Cell proliferation was determined with BrdU (5-bromo-2′-deoxyuridine) incorporation. Apoptosis was examined with TUNEL (transferase-mediated dUTP nick end-labeling). Morphometry was performed on histologic sections.

Results: Coarctation produced hypertension in the proximal aorta, 118 ± 9 mm Hg versus 94 ± 6 mm Hg in controls (P < .002). Both messenger RNA and protein levels of transforming growth factor (TGF)-β1 and TGF-β3 were increased (P < .005 vs controls). Messenger RNA and protein of Bcl-xS and Fas ligand, known as proapoptotic factors, were both reduced after coarctation (P < .005 vs controls). There was increased BrdU incorporation at 3 days and 1 and 2 weeks (P < .001 vs controls). There were no remarkable changes in the apoptosis rate until 4 weeks later.

Conclusion: Cell proliferation was stimulated at 3 days, and apoptosis was halted until 4 weeks. These changes were associated with upregulation of TGF-β1 and downregulation of Bcl-xS and Fas ligand gene expression. These findings suggest that a coordinated regulation of cell proliferation and cell death contributes to arterial remodeling in response to acute sustained elevation of blood pressure. Cell proliferation precedes apoptosis by 2 weeks in this procedure.

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Arteries remodel in response to hemodynamic changes, including hypertension.1-6 Hypertension not only induces arterial remodeling but also promotes atherogenesis.7,8 Cell proliferation and apoptosis are thought to play an important role in the balance of cellular and extracellular volumes of the arterial wall during the remodeling.9-12 However, it has also been shown in hypertensive arteries that smooth muscle cell (SMC) replication is increased without counterbalance by increased apoptosis, which results in medial thickening of arteries and arterioles.13

Many growth-modulating factors are involved in remodeling. Transforming growth factor (TGF)-β1 is a pleiotropic factor expressed within vascular cells that regulates cellularity in a tissue-specific manner. It can exert either proapoptotic or antiapoptotic effects, depending on the cell type, circumstances, or both. The responses and roles of TGF-β1 in a variety of in vivo and in vitro conditions have been reported.14-18 TGF-β1 could contribute to the development of hypertension or vascular hypertrophy in spontaneous hypertensive rats.19 It has also been reported that TGF-β1 further potentiates the induction of apoptosis in endothelial cells (ECs) in serum-free media.14 Furthermore, TGF-β1 can prevent apoptosis through a mechanism that relies on early induction and phosphorylation of c-Jun.20 Thus, the role of TGF-β1 in vessel remodeling is still not well defined.

Apoptosis has been shown to be associated with a variety of physiologic and pathologic conditions in the cardiovascular system. For instance, apoptosis has been shown in postnatal heart morphogenesis,21 in postpartum arterial remodeling, in the regulation of endothelial turnover, and in pressure-overloaded hypertrophy.22-24 Apoptosis is modulated by variety of proapoptotic and antiapoptotic factors. For example, Bcl-xS is a proapoptotic factor, and increased induction of Bcl-xS expression has been associated with SMC apoptosis in the intimal thickening of balloon-injured carotid arteries.25 However, antisense Bcl-x oligonucleotide induced apoptosis and prevented arterial neointimal formation in murine cardiac allografts.26 Thus, the specific role of apoptosis in vessel remodeling remains poorly defined.

In the current study we aimed to test the hypothesis that both apoptosis and cell proliferation are equally essen-
tial in vessel remodeling, with an experimental acute hypertension model. The results showed that the two processes coexisted during aortic remodeling in response to hypertension and that cell proliferation preceded apoptosis by 2 weeks.

MATERIALS AND METHODS

Aortic coarctation model. A midthoracic aortic coarctation was created in rats (250-300 g). The procedure was similar to our previous model in rabbits. The study was approved by the Institutional Review Board, and the animal care complied with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission of Life Science, National Research Council. Washington: National Academy Press; 1996). Surgical procedures were conducted with the rats under general anesthesia and in sterile conditions. Rats were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. After trachea intubation, the animals were kept under the control of a respirator with a tidal volume of 2.5 mL at a frequency of 90 times per minute. Left thoracotomy was performed at the fifth intercostal space to expose the aorta, and a piece of surgical silk suture (#0) was placed surrounding the midthoracic segment. A 20-gauge needle with an outer diameter of 0.9 mm was placed on the top of the aortic segment. The aorta and the needle were tied together with the suture. The needle was then retrieved immediately, leaving a 0.9-mm lumen diameter at the constriction site. The chest was closed with air suction. The tracheal tube was removed after the animal woke up. Before the rat was humanely killed, blood pressure was measured through catheters introduced in the carotid and femoral arteries. Sham-operated animals were operated on in the same procedure as for the coarctation except that the suture was positioned and tied up, but there was no constriction of the aorta.

Time course. Acute aortic hypertension was induced in a time course manner, 1 and 3 days and 1, 2, and 4 weeks (n = 3 each). An equal number of sham-operated and age-matched animals served as controls. Two sets of animals were used. One set was for the extraction of total tissue RNA and protein and the other for perfusion fixation. For frozen tissue, animals were intravenously injected with lethal doses of pentobarbital. The aorta was dissected, and surrounding fatty tissue was carefully removed. A 2-cm segment, 5 mm proximal to the coarctation, was immediately taken and frozen in liquid nitrogen for extraction of total tissue RNA and proteins with Trizol Reagent (Life Technologies, Inc, Rockville, Md). For histologic examination and detection of cell proliferation and apoptosis, the animals were perfusion fixed with 4% paraformaldehyde at room temperature and at a pressure of 100 mm Hg for 30 minutes. The aorta was then taken and further fixed in 4% paraformaldehyde. A cross-sectional ring 5 mm proximal to the coarctation was trimmed for frozen sections.

Ribonuclease protection assay. Gene expression of cell growth and apoptosis-related cytokines was studied in the hypertensive aorta segment proximal to the coarcta
tion. Ribonuclease protection assay (RPA) was performed to obtain relative messenger RNA (mRNA) levels. Nonradioactive RNA probes were transcribed with two sets of multiple probe templates, rCK-3 and rAPO-1, (Pharmingen, San Diego, Calif). An in vitro RNA transcription kit (Promega, Madison, Wis) was applied with the addition of biotin-16-UTP for labeling the probes (Roche, Indianapolis, Ind). The sensitivity and specificity of the probes were carefully tested before we started the experiment. An RPA-III kit (Ambion, Austin, Tex) was used for RPA following the manufacturers protocols. The resulted films were scanned, and densitometry was performed with NIH Image program version 1.62 (National Institutes of Health, Bethesda, Md). The relative values of protected probe fragments were expressed as a ratio of their densitometry values over those of GAPDH (glycer
aldehyde-3-phosphate dehydrogenase) in the same lane. The mean and SD were calculated for each time point.

Cell proliferation assay. Incorporation techniques of BrdU (5-bromo-2’-deoxyuridine) were used to assess DNA synthesis for cell proliferation. Three injections of BrdU (Sigma, St Louis, Mo), 50 mg/kg body weight, were administered intraperitoneally to the animals 20, 6, and 1 hours before they were humanely killed. Immunodetection of BrdU incorporation was performed on frozen sections with a detection kit (Roche). The cell type of BrdU-incorporated cells was assessed histologically. In addition, an antibody against α-actin (Enzo Diagnostics, Inc, Farmingdale, NY) was used to confirm SMCs in the media. The number of BrdU-labeled nuclei was counted from each whole cross section. The mean and SD were calculated from three animals for each time interval, and the cell proliferation rate was expressed as a number of labeled nuclei per section.

Western blot analysis. Protein samples in 2× sodium dodecylsulfate loading buffer were fractionated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis at 100 V until the dye front reached the bottom of the gel. The proteins were transferred onto 0.45-µm pore nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 40 V for 45 to 60 minutes. The membranes were blocked with 5% nonfat milk and incubated at room temperature for 1 hour with anti–TGF-β1 polyclonal antibody at a 1:200 dilution, anti–TGF-β3 polyclonal antibody at a 1:1000 dilution, anti–Bcl-xS/L polyclonal antibody at a 1:500 dilution, and anti–Fas ligand (FasL) polyclonal anti-

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<th>Mean aortic blood pressure (mm Hg)</th>
<th>Carotid</th>
<th>Femoral</th>
<th>Gradient</th>
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<tr>
<td>Controls (n = 3)</td>
<td>94 ± 6</td>
<td>93 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Coarctated (n = 18)</td>
<td>118 ± 9*</td>
<td>98 ± 10</td>
<td>19 ± 8*</td>
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*P < .002 compared with controls.
body at a 1:500 dilution (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). After extensive washes with phosphate-buffered saline, the blots were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (1:1000) for 1 hour at room temperature. The blots were washed three times in phosphate-buffered saline, and the protein complexes were detected with enhanced chemiluminescence detection reagents according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc, Piscataway, NJ). The data presented represent results from three animals in each of the groups. Protein samples from each animal were fractionated on more than one gel, in duplicate or triplicate.

**Apoptosis detection.** The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method was used on frozen sections to demonstrate apoptosis in SMCs and ECs. An in situ cell death detection kit (Roche) was used for TUNEL with modifications. Nuclei of apoptotic cells were thus labeled with fluorescein, and the sections were counterstained with propidium iodide and observed under laser-scanning confocal microscope (Bio-Rad MRC 1024, Hercules, Calif). Negative controls were included by omitting the labeling buffer. Positive controls were obtained by digestion of the sections with deoxyribonuclease I for 10 minutes at room temperature before the detection procedure. Labeled nuclei were counted per section. The mean and SD of each time point were obtained from three animals for statistical analysis. In addition, transmission electron microscopy (TEM) was used to confirm the morphologic changes of apoptotic cells. TEM sample preparation followed routine procedures of our laboratories.3,4

**Statistical analysis.** Data were presented as mean ± SD. Single factor analysis of variance and the Bonferroni test were used for multiple comparisons. StatView software version 5.0 (SAS Institute, Inc, San Francisco, Calif) was used for the statistical analysis. The difference was considered significant if \( P \) was less than .0033.

**RESULTS**

**Blood pressure measurements.** Midthoracic aortic coarctation produced an elevation of blood pressure in the
proximal aorta (Table). There were blood pressure gradients across the coarctation, ranging from 15 to 25 mm Hg.

**Gene expression of cell growth–related cytokines.** With the multiple probes of rCK-3, RPA revealed an upregulation of TGF-β1 and TGF-β3 with an increase in relative mRNA levels at 1 and 3 days ($P < .0033$ vs controls) (Fig 1, *left panels*). TGF-β1 gene expression was increased at 1 day and gradually declined thereafter, returning to baseline levels at 2 weeks. TGF-β3 was highly expressed at 1 and 3 days and returned to baseline levels at 2 weeks. Both TGF-β1 and TGF-β3 reached their expressive peaks at 1 day with an increase of 1.9-fold and 2.3-fold, respectively, compared with controls. Western blot analysis revealed a similar trend for corresponding protein expression. The protein levels of TGF-β1 and TGF-β3 increased significantly at 3 days (Fig 1, *right panels*).

**Gene expression of apoptosis-related cytokines.** With the multiple-probe of rAPO-1, RPA showed that relative mRNA levels of Bcl-xS and FasL, known as proapoptotic factors, were decreased at 1 day and remained. At 2 weeks Bcl-xS returned to control level, and at 4 weeks FasL returned to higher than control level. The remarkable change was that FasL expression rebounded to higher than control level by 4 weeks ($P < .0033$ vs controls) (Fig 2, *left panels*). Western blot analysis showed a similar trend for corresponding protein expression (Fig 2, *right panels*).

**Cell proliferation was induced after coarctation.** Morphologic observation showed an increased cellularity, including ECs and SMCs (Fig 3). The cells in the media were SMCs as confirmed by staining with the antibody against muscle α-actin (data not shown). BrdU-incorporated nuclei were observed in the media, along the endothelial line and in the adventitia (Fig 4). The incorporation rate was increased at 3 days and at 1 and 2 weeks ($P < .001$ vs controls) (Fig 5).

**Cell apoptosis rate was increased by 4 weeks.** Apoptosis of vascular cells was detected with the TUNEL method in all animals (Fig 6). Although the apoptosis rate for the coarctated animals was not remarkably different in the early 2-week period as compared with controls, there was an increased apoptosis rate by 4 weeks ($P < .0033$ compared with controls, Figs 6 and 7). Ultrastructural study with TEM confirmed characteristic features of apoptotic cells. In general, the nuclei appeared to be condensed...
Fig 3. Cellularity of ECs and SMCs was within normal population (Ctrl). Starting from 1 day, cellularity of ECs and SMCs increased, and they appeared crowded (1D-4W). Ctrl, Controls; D, day; W, week.

Fig 4. BrdU-incorporated nuclei were labeled dark blue by the immunodetection mediated by alkaline phosphatase. All nuclei were counterstained pink with fast red. ECs and SMCs showed BrdU incorporation. Higher power view of the framed zone on 1-W panel is shown on far right side. Ctrl, Control; D, day; W, week.
and fragmented, and finally, the cytoplasm became condensed, too (data not shown).

**Lumen diameter and wall thickness.** Morphometry on histologic sections showed no statistically significant difference in lumen diameter. However, wall thickness was increased by 1 week (0.104 ± 0.001 mm vs 0.075 ± 0.001 mm for controls), and such thickness was maintained at 2 and 4 weeks (Fig 8).

**DISCUSSION**

This study showed that both cell proliferation and apoptosis were involved in vessel remodeling in response to acute elevation of blood pressure. Cellular proliferation included increase in cellularity of both SMCs and ECs. BrdU incorporation analysis demonstrated that the proliferation rate peaked at 1 week after coarctation, suggesting that the response was characterized by cell proliferation. Along with the cellular proliferation, TGF-β1 and TGF-β3 gene expression was upregulated. The relative mRNA levels for the two cytokines were increased as early as 1 day and remained high at 3 days before returning to control levels by 2 weeks. Changes of corresponding protein levels had a similar trend. It seems that TGF-β gene upregulation was an immediate response followed by cell proliferation. Our results support the role of TGF-β1 and TGF-β3 in cell proliferation.

Apoptosis was detected with TUNEL in all animals, and ultrastructural features of apoptotic cells were confirmed with TEM. The apoptosis rate was not significantly increased until 4 weeks after coarctation. This finding was associated with an initial decrease in mRNA and in protein levels of the proapoptosis genes, Bcl-xS and FasL, and a rebound of them by 4 weeks. The mechanism that down-regulation of Bcl-xS and FasL gene expression was not associated with a decline in the apoptosis rate during the early 2 weeks remains to be clarified. However, by 4 weeks, the gene expression for Bcl-xS returned to control level and rebounded to higher than control level for FasL, which was attested by the increase in apoptosis rate at 4 weeks. These results showed that cell proliferation preceded apoptosis during the vessel remodeling in response to high blood pressure.

Cell proliferation has been documented in vessel remodeling in response to hypertension. The genes of the TGF-β family play multiple roles in vessel remodeling in response to hemodynamic changes. We have shown that TGF-β1 gene expression was increased at 7 days after flow-induced artery dilation. TGF-β may play a role in modulating cell proliferation and gene expression of extracellular matrix. In the current study we have observed the increased BrdU incorporation across the wall. We have previously demonstrated that gene expression of collagen types I and III was increased. It is therefore speculated that hypertension may upregulate the TGF-β gene expression, which modulates cell proliferation and gene expression of collagen. The outcome of these regulations may be to maintain the strength of the vessel wall to limit its enlargement.

Apoptosis is involved in tissue morphogenesis and homeostasis. It is the essential counterpart of cell replication. Apoptosis was postulated that remodeling during atherogenesis or remodeling in hypertension might be mediated by coordinated action of cell proliferation and apoptotic cell death. Indeed, hypertension can induce marked apoptosis in the vessel media and in target organs of hypertension. Little is known about how apoptosis coordinates with cell proliferation during the remodeling in response to hypertension. Our findings demonstrated that the apoptosis rate increased at 4 weeks after the elevation of blood pressure, whereas cell proliferation reaction started as early as 3 days and peaked at 1 week. This time differentiation suggests that cell proliferation is the immediate response to hypertension to ensure a reinforced vessel wall, which may prevent the vessel wall from weakening.

**Fig 5.** BrdU incorporation rate (mean ± SD) was expressed as number of labeled nuclei of ECs and SMCs per section obtained from three animals of each time interval. Incorporation rate increased at 3 days (52 ± 11) after coarctation. It peaked at 1 week (73 ± 7), dropped to 31 ± 4 at 2 weeks, and returned to control level by 4 weeks (4 ± 1). *P < .001 compared with controls (4 ± 2).
Fig 6. Cell apoptosis detection with TUNEL. Sections were observed under a laser-scanning confocal microscope. All nuclei were counterstained red with propidium iodide, and nuclei of apoptotic cells were labeled green with fluorescein. ECs and SMCs showed positive labeling. Elastic lamellae appeared light green because of their auto fluorescence. By 4 weeks, number of labeled nuclei seemed to be increased. Ctrl, Control; D, day; NC, negative control; PC, positive control; W, week.
Nevertheless, apoptosis would further help remodeling. Although the increase in the apoptosis rate seemed to be associated with the increase in wall thickness at 4 weeks of coarctation, the actual contributor to the increased wall thickness was the accumulation and remodeling of newly synthesized collagens and other extracellular matrix. Apoptosis is a cytokine-regulated form of cell death. The expression and cascade activation of proteins and proteinases appear as key events mediating apoptosis. For instance, the protein Bcl-2 is expressed in SMCs. Constitutive expression of Bcl-2 suppresses apoptosis almost universally with regard to the cell types. On the other hand, the Bcl-2–related gene, Bcl-x, encodes both positive and negative regulators of apoptosis depending on alternative mRNA splicing. Bcl-xS is a proapoptosis protein. We showed that Bcl-xS gene expression returned to control levels at 4 weeks after reduction during the earlier weeks. It has been demonstrated that both ECs and vascular SMCs express Fas (CD95) (a member of the tumor necrosis factor family of receptors), and ECs also express FasL, suggesting that Fas-mediated apoptosis of vascular SMCs may also occur. Indeed, mice lacking components of the Fas/FasL signaling complex have arteritis. In our study, FasL gene expression showed a similar pattern to Bcl-xS, and moreover, the gene expression of FasL was higher than that of controls by 4 weeks, indicating that FasL may play a more triggering role in the apoptosis event. It may not just be a coincidence that the apoptosis rate was increased by 4 weeks.

Fig 7. Apoptosis rate (mean ± SD) was expressed as number of TUNEL positive nuclei per section obtained from three animals of each interval. Apoptosis rate was not remarkably changed until 4 weeks (25 ± 6). *P < .0033 compared with controls (10 ± 6). D, Day; W, week.

Fig 8. Wall thickness was increased by 1 week, and such thickness was maintained at 2 and 4 weeks. *P < .002 as compared with age-matched controls. D, Day; W, week.
REFERENCES


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