Degradation of the Internal Elastic Laminae in Vein Grafts of Rats with Aortocaval Fistulae

Potential Impact on Graft Vasculopathy

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The internal elastic lamina (IEL) of vein grafts may be modified when exposed to arterialized hemodynamics. We investigated changes of the IEL in the inferior vena cava (IVC) of rats with aortocaval fistulae (ACF). In the IVC of ACF rats, both a markedly increased flow velocity and a mildly increased pressure were demonstrated. In the lower segment where hemodynamic changes were prominent, neointimal hyperplasia was prominently found. The IEL of the IVC in sham-operated rats, observed by confocal microscopy, was composed of parallel elastic fibers. In ACF rats, the IEL degenerated progressively after surgery. The elastic fibers were stretched and gradually became sparse, a change that was more prominent in the lower segment. Eight weeks after surgery, the IEL hardly existed in some areas of the lower segment. Electron microscopy revealed decreased densities and diameters of elastic fibers. Reverse transcriptase-polymerase chain reaction analysis revealed an up-regulation of potent elastases, cathepsins K and S, and matrix metalloproteinase-2 in the IVC of ACF rats. Results of immunohistochemical studies localized cathepsin expression predominantly to the luminal endothelium lining the IEL, suggesting involvement of elastinolysis in the degradation of the IEL. We demonstrated the degradation of the IEL in the vein graft of ACF rats, especially in the segment exposed to prominent hemodynamic changes. IEL degradation may contribute to the development of neointimal hyperplasia in vein grafts. (*Am J Pathol* 2009, 174:1837–1846; DOI: 10.2353/ajpath.2009.080795)

Vein grafts are the major conduits for bypass surgery in patients with coronary or peripheral arterial diseases. In uremic patients, vein grafts are also the most common vessels used for creation of the arteriovenous fistula for hemodialysis access. After anastomosis with the artery, the vein graft is exposed to abrupt hemodynamic changes, such as increased flow,¹ shear stress,^{2,3} and tensile stress.⁴ These mechanical stresses have been demonstrated to regulate the gene expression and function of various vascular cells, especially the endothelial cells^{5,6} and smooth muscle cells (SMCs),^{7,8} which consequently contribute to the development of graft vasculopathy. In addition to the cellular component, the extracellular matrix may also be modified by these mechanical stresses, directly or indirectly. The internal elastic lamina (IEL) is a vascular structure composed of elastic fibers and forms a barrier between the intima and media. Its degradation has been found to be associated with the formation of arterial aneurysms^{9,10} and development of neointimal hyperplasia in balloon-injured artery.^{11,12} In vein grafts, the IEL may also be modified and contribute to the pathogenesis of graft vasculopathy.

This study investigated the remodeling of IEL of inferior vena cava (IVC) in rats with aortocaval fistula (ACF), a model that mimics the hemodynamics of vascular access for hemodialysis in uremic patients. The expressions of elastogenesis- and elastinolysis-associated molecules, which may be involved in IEL remodeling, were also investigated.

Materials and Methods

Rat Model of ACF

The ACF model was created in adult male Sprague-Dawley rats (300 to 350g). After anesthetization with ket-

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Figure 1. Photograph of the rat model of ACF to illustrate the anatomical site of ACF creation and transaction site that divided the IVC into the upper and lower segments. Abd Ao, abdominal aorta.

amine (0.2 ml/100 mg i.p.), the abdominal cavity was opened. The IVC and aorta were exposed and clamped right below the renal artery proximally and right above the iliac bifurcation distally. An 18-gauge needle was used to puncture the lateral wall of the abdominal aorta, right superior to the distal clamped site. The needle was advanced to cross the opposite aortic wall toward the IVC and then penetrate the neighboring wall of the IVC cautiously to avoid puncturing the opposite wall. Finally, the needle was withdrawn gently; the entry point was sealed with cyanoacrylate glue (Vetbond 3M, St. Paul, MN). The patency of the fistula was confirmed by pulsation and color change in the IVC, resulting from the shunt of oxygenated blood from the aorta. The sham operation was done by simply puncturing the lateral wall of IVC, which served as a control to rule out the artificial change of IEL caused by puncturing of the vessel. When harvested, the IVC was transected equally into the upper and lower segments. The anatomical location of ACF creation and the transaction site that divided the IVC into the upper and lower segments are illustrated in Figure 1. The specimens for general histology, immunohistochemistry, and confocal and electron microscopic studies were harvested with the animals perfusion-fixed with 3% paraformaldehyde in phosphate-buffered saline at 100 mm Hg for 10 minutes. All of the animal procedures were performed according to the guidelines of the committee on animal research at Chang Gung Memorial Hospital.

Duplex Scan to Assay IVC Flow

Duplex scan (Acuson, Aspen, Mountain View, CA) was performed to assay IVC flow. B-mode and Doppler imaging were obtained using a high-resolution linear transducer at a frequency of 15 MHz. After euthanization and laparotomy, the IVC was exposed and the transducer placed over the IVC as lightly as possible to avoid compressing the vessel, as guided by the B-mode image. The IVC flow velocity was recorded along the entire IVC segment.

Measurement of IVC Pressure

Two weeks after surgery, rats were anesthetized with urethane (1.25 g/kg, i.p.). A polyethylene cannula (PE 50) filled with heparinized saline (25 IU/ml) was inserted into the right jugular vein and advanced further to the lower

IVC to measure the intraluminal pressure. The cannula was connected to a pressure transducer (MLT0380/D; ADInstruments Pty. Ltd., Bella Vista, Australia) and the signals were amplified by a bridge amplifier (QuadBridge Amp, ADInstruments). Signals from these amplifiers were digitalized and analyzed by Chart 5.4.2 software (ADInstruments).

Measurement of IVC Circumference

IVC circumference was measured *ex vivo*. Perfusion-fixed IVCs were opened longitudinally and mounted. Maximal widths of the upper and lower segments of each IVC specimen were measured by two investigators blind to the animal experiments.

Tissue Preparation for Confocal Microscopy

Perfusion-fixed IVCs were harvested and the adventitia was trimmed gently to avoid vessel injury. The vessel was opened longitudinally and mounted with the luminal side facing up. IVC samples were then examined using a confocal laser-scanning microscope (TCS SP; Leica, Wetzlar, Germany). The optical sectioning properties of the confocal microscope were applied to investigate the IEL structure, which gave the autofluorescence of elastin. The image obtained consisted of projection views of consecutive optical sections taken at 0.8- μ m intervals through the entire thickness of signal of elastic fibers in each observed field. The feasibility of observing the IEL of the vessel by confocal laser-scanning microscopy has been verified in a previous study.^{13,14}

Electron Microscopy

Perfusion-fixed IVCs were dissected, opened longitudinally and postfixed in 1% glutaraldehyde. Specimens were then incubated in 1% osmium tetroxide, dehydrated through an ethanol series, and finally embedded in Epon 812. Sections (80 nm) were cut and stained with uranyl acetate and lead citrate. Sections for observing the fragmentation of elastic fibers were stained with tannic acid-uranyl acetate followed by the lead citrate, a staining technique that has been shown to give precise and highly specific location of elastin aggregates and elastic fibers.^{15,16} This staining method may confirm that the fragmentation of elastic fibers is not caused by inadequate staining. The specimens were examined in a Hitachi (Tokyo, Japan) H7500 electron microscope.

Immunohistochemistry Study

IVC specimens harvested at 2 weeks were prepared for immunofluorescent histochemistry as described previously.¹⁷ Briefly, the frozen sections (8 μ m thick) of the IVC specimens were blocked in 0.5% bovine serum albumin for 15 minutes, and incubated with rabbit anti-human cathepsin K (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human cathepsin S (1:200, Santa Cruz Biotechnology), rabbit anti-human matrix metallo-

proteinase (MMP)-2 (1:100; Neomarkers, Fremont, CA), goat anti-mouse CD68 (1:100, Santa Cruz Biotechnology), or goat anti-human CD3 (1:200, Santa Cruz Biotechnology) antibody at 37°C for 1 hour. Samples were then treated with CY3-conjugated secondary antibody (1:500) at room temperature for 1 hour. Finally, samples were stained with Hoechst stain for 5 minutes to visualize cell nuclei. Double-labeling immunofluorescence was performed to identify the cell types that express cathepsin K, cathepsin S, and MMP-2. The goat anti-mouse CD31 (1:500, Santa Cruz Biotechnology), monoclonal anti-human smooth muscle (SM) α -actin (1:100; Sigma, St. Louis, MO) and goat anti-human CD3 (1:100, Sigma) antibodies were used to identify the endothelial cell, SMC, and lymphocyte, respectively. The immunostaining for the elastases was performed as described above with a Cy3-conjugated secondary antibody used. Then the sections were washed copiously with phosphate-buffered saline and exposed to the second primary antibody for 1 hour. Then corresponding secondary antibody conjugated to Cy5 was applied.

Reverse-Transcription (RT) Semiquantitative Polymerase Chain Reaction (PCR)

RNA was isolated from IVC by acid phenol extraction. RNA was reverse-transcribed using Superscript II reverse-transcriptase (Gibco-BRL, Life Technologies GmbH, Eggenstein, Germany). Two μ l of the cDNA sample was added to a mixture of 10 μ l of PCR buffer [200 mmol/L Tris-HCl, pH 8.4, 500 mmol/L KCl, 3 μ l MgCl₂ (50 mmol/L), 2 μ l dNTP (10 mmol/L), 1 μ l each of 5' and 3' primer (25 μ mol/L), 5 U TaqDNA polymerase, and 80 μ l autoclaved, distilled water]. PCR conditions were as follows: 94°C for 45 seconds, 62°C for 1 minute, and 72°C for 1 minute. Finally, extension was performed for 10 minutes at 72°C. Products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. Primer sequences were as follows: lysyl oxidase forward: 5'-CTGCTGCTGCGTGACAAC-3', lysyl oxidase reverse: 5'-GACGGCGAGAAACCAACT-3', fibrillin 1 forward: 5'-TACCGCCTCACCTCCACA-3', fibrillin-1 reverse: 5'-CCCAGCCTTCTCCCTTCA-3', tropoelastin forward: 5'-TGGAGCCCTGGGATATCAAG-3', tropoelastin reverse: 5'-GAAGCACCAACATGTAGCAC-3', cathepsin S forward: 5'-AAGTACGGGAATAAAGGC-3', cathepsin S reverse: 5'-AGGAAGAAGGAGGAATGG-3', cathepsin K forward: 5'-CTCTGAAGACGCTTACCC-3', cathepsin K reverse: 5'-ATTATCACGGTCGCAGTT-3', cystatin C forward: 5'-GTAAGCGAGTACAACAAGGG-3', cystatin C reverse: 5'-GATCTGGAAGGAGCAGAGTA-3', MMP-2 forward: 5'-GATAACCTGGATGCTGTCTG-3', MMP-2 reverse: 5'-CTTCCAACTTCACGCTCT-3', tissue inhibitor of metalloproteinase (TIMP)-2 forward: 5'-ACAG-GCGTTTTGCAATGCAG-3', TIMP-2 reverse: 5'-CGCGC-AAGAACCATCACTTC-3'.

Band intensities were documented by a digital gel-imaging system. All data were normalized against GAPDH mRNA level, which was used as an internal standard. The mRNA levels of investigated genes in each

time point were compared between both sham-operated and ACF groups.

Measurement of the Density and Diameters of Elastic Fibers

The density of elastic fibers of the IEL was defined as the number of fibers in each electron microscopic field at a magnification of $\times 4000$. Fifteen electron microscopic fields of each segment were counted and the mean densities were calculated. The maximal diameters of 50 elastic fibers in cross-sectional views of each segment were measured manually and mean diameters were calculated.

Cathepsin Activity Assay

Cathepsin K and S activities were assessed using activity assay kits (Biovision Research Products, Mountain View, CA). The kits are fluorescence-based assays using the preferred cathepsin K or S substrate sequence labeled with amino-4-trifluoromethyl coumarin. Briefly, the synthetic substrates were incubated with whole tissue lysate of IVCs in reaction buffer at 37°C for 2 hours. The specificity of the assay was confirmed by incubating samples in the presence of cathepsin-specific inhibitors. The released fluorescence activity was measured at 400-nm excitation filter and 505-nm emission filter. Relative activity was calculated by dividing the fluorescence activity by that of the sham-operated group.

Gelatin Zymography

Electrophoresis was performed with 20 μ g of whole tissue lysate of IVCs on a 10% polyacrylamide/sodium dodecyl sulfate gel containing 1 mg/ml of gelatin. Sodium dodecyl sulfate was removed by washing in 2.5% Triton X-100 for 1 hour at room temperature before the enzyme reaction. The gel was incubated overnight at 37°C in enzyme buffer containing 50 mmol/L Tris, pH 7.5, 200 mmol/L NaCl, 5 mmol/L CaCl₂, and 0.02% Brij-35. Area of gelatin degradation, identified as MMP activity, appeared as distinct white band after staining the gel with 0.5% Brilliant Blue G-250. The intensities of both pro- and active MMP-2 were documented by a digital gel-imaging system. Relative activity was calculated by dividing the densitometric value by that of the sham-operated group.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Differences between groups were evaluated by either one-way analysis of variance or Student's *t*-test, as appropriate. Analysis of variance trend analysis was used to test the time-dependent increase of the IVC circumferences. Linear regression analysis was used to evaluate the independent effect of ACF creation on the circumferences of IVC, adjust for timing and segment of IVC. Values were considered significant at $P < 0.05$.

Figure 2. Hemodynamic assays and circumference measurement for IVC in sham-operated and ACF rats. **A:** Duplex scan assay to detect the flow velocity of IVC. **B:** Pressure tracing of IVC. **C:** Comparison of the mean peak flow velocity of IVC between both groups. **D:** Comparison of the mean peak pressure of IVC between both groups. **E:** Time-dependent change of circumference of IVC.

Results

Hemodynamic Assay and Circumference Measurement

IVC Flow Velocity

IVC flow velocities were measured by duplex scan at 2 weeks ($n = 3$ for each group) as demonstrated in Figure 2A. In the sham-operated rats, a normal central venous flow pattern, reflecting atrial pulsation, was detected. The flow velocity was low and constant throughout the entire segment with a mean peak velocity of 0.09 ± 0.04 m/sec. In the ACF group, turbulent flow with highly variable velocity was detected at the lower IVC segment near the aortocaval anastomosis. In the other portion of the lower IVC segment, the flow was pulsatile with a high velocity and wide velocity spectrum indicating a turbulent flow. In the upper segment, the flow remained to be pulsatile, but the velocity was lower and varied with respiration. The mean peak velocities of the lower and upper segments

were 4.7 ± 0.22 m/second and 0.79 ± 0.15 m/second, respectively, both were significantly higher than that of the sham-operated group ($P < 0.001$ for both). When compared between the upper and lower segments of the ACF group, the flow velocity was significantly higher in the lower segment ($P < 0.001$) (Figure 2B).

IVC Pressure

The pressure tracing for IVC was performed at 2 weeks as demonstrated in Figure 2C ($n = 3$ for each group). In the sham-operated group, pressure tracing showed a normal central venous pressure waveform. The pressure was low and remained constant through the entire IVC segment with a mean peak pressure of 4.1 ± 0.9 mm Hg. In the ACF group, the IVC pressure was pulsatile and fluctuated with the respiration. The maximal pressure was detected near the aortocaval anastomosis. The mean peak pressure of the lower segment was significantly higher than that of the upper one (13.3 ± 2.3 mm Hg versus 5.0 ± 1.2 mm Hg, $P < 0.001$). Compared with the sham-operated group, the mean peak pressure of the lower segment of ACF group was significantly higher ($P < 0.001$). In contrast, the mean peak pressure of the upper segment was not different from that of the sham-operated group ($P = 0.23$) (Figure 2D).

IVC Circumference

The maximal circumferences of the upper and lower IVC segment were measured separately at 1, 2, 4, and 8 weeks ($n = 3$ for each time point in both groups) and presented in Figure 2E. In the sham-operated group, the maximal circumferences significantly increased after surgery in the upper, but not the lower, IVC segment ($P < 0.001$ and $P = 0.115$, respectively). In the ACF rats, both the upper and lower IVC segments increased after surgery ($P < 0.001$ and $P = 0.019$, respectively). Linear regression analysis showed that AVF creation is an independent predictor of larger maximal circumferences of IVC ($P = 0.003$).

General Histology

The general histology of the IVC in sham-operated rats showed a thin vascular wall without any neointimal lesions throughout the entire segment at any time points (Figure 3A). In the upper IVC segment of ACF rats, the general histology was not different from that of the sham-operated rats at any time point (Figure 3B). In contrast, some early focal neointimal lesions composed of few cells budding into the vascular lumen were observed in the lower IVC segment of ACF rats as early as 2 weeks after surgery (Figure 3C). Four weeks after surgery, larger papillomatous neointimal lesions were found. Some particularly large lesions were found to be in contiguity with the opposite vessel wall with necrotic core in some area (Figure 3D). These findings were consistent with a previous study using the same animal model.¹⁸ One interesting observation is that no neointimal tissues were found at

Figure 3. Representative photomicrographs of IVC of the sham-operated and ACF rats. **A:** The IVC of sham-operated rat at 8 weeks showed normal venous histology. **B:** The upper IVC segment of ACF rat at 8 weeks was indistinct from that of the sham-operated rats. **C:** In the lower IVC segment of ACF rats, early neointimal lesions (arrows) were observed in the lower IVC segment at 2 weeks. **D:** A large neointimal lesion (NH) with central necrosis (arrows) was observed in the lower IVC of ACF rat at 8 weeks. **E and F:** Infiltration of macrophages and T lymphocytes, identified by anti-CD68 and anti-CD3 antibodies, in IVC of ACF rats at 2 and 4 weeks. Macrophages were found in tissue surrounding the aortic puncture site but not in IVC (arrows in **E**). Infiltration of T lymphocytes was observed in the neointimal lesions (arrows in **F**). **G and H:** Confocal microscopic images of the IVC. The IEL was visualized by its autofluorescence (arrowheads). In the sham-operated rat at 8 weeks, the IEL was observed as a waving line. **G:** In the lower IVC segment at 8 weeks, disruption of the IEL was present, from which a cluster of cells emerged (arrows, **H**). **A–D,** H&E stain; **E–H,** Hoechst stain to visualize the cell nuclei. Original magnifications: $\times 20$ (**A–D**); $\times 630$ (**E, F**); $\times 400$ (**G, H**).

any time point in the upper segment. Immunohistochemistry was done to detect the infiltration of inflammatory cells in IVC at 2 and 4 weeks after operation. The anti-CD68 and anti-CD3 antibodies were used to identify the macrophages and T lymphocytes, respectively. Observation by confocal scanning microscope revealed that the anti-CD68 signals were hardly detectable at intima, including neointimal lesions, or media of IVC at both time points. Small clusters of anti-CD68 signals were found at tissue around the aortic puncture site at 2 weeks (Figure 3E). Isolated anti-CD3 signal was found in the neointima at 2 and 4 weeks (Figure 3F). The signal was not detected in the media at 2 or 4 weeks. These data indicated infiltration of T lymphocytes in neointima but not media, and no remarkable infiltration of macrophage in intima or media of IVC of ACF rats at 2 or 4 weeks after operation. When observed by confocal microscopy, the IEL of IVC in the sham-operated rats was visualized by its autofluorescence as a waving line (Figure 3G). In the ACF rats, interruption of IEL was found in some foci of the lower segment. Clusters of cells were found to emerge from the foci where IEL disrupted, suggesting migration of medial SMCs to the intima through these defects (Figure 3H).

Structural Remodeling in the IVC of ACF Rats

The perfusion-fixed IVC specimens harvested at 10 minutes, and 2, 4, and 8 weeks were observed *en face* using confocal laser-scanning microscopy to investigate the structural changes of the IEL. Different from the fenestrated sheet-like structure of IEL of the artery as reported previously,^{13,14} the IEL of IVC in the sham-operated rats was composed of elastic fibers arranged in parallel and oriented obliquely to the longitudinal axis of the IVC (Figure 4, A and B). The fibers were interconnected by fibers of smaller diameter. Similar IEL structure was observed in IVC of normal rats (data not shown). The IEL remained without obvious changes at any time points. In the ACF group, the IEL was grossly intact at 10 minutes after surgery except a hole with a clear-cut margin caused by puncture (Figure 4C). These findings confirmed that the surgery did not cause unexpected damage to the IEL. At 2 weeks, the parallel-arranged elastic fibers of IEL were torn apart from each other. This pattern was found throughout the entire IVC and was more prominent in the lower segment (Figure 4, D and E). At 4 weeks, the IEL of the upper segment remained without further changes. However, the IEL in the lower segment further degraded

Figure 4. Representative confocal microscopic images of the IEL of IVC. **A** and **B:** In the sham-operated rats, the IEL was composed of parallel-arranged elastic fibers. The general structures of the IEL were indistinct between the upper (**A**) and lower (**B**) segments. **C–H:** In the ACF rats, the IEL remained intact at 10 minutes after surgery except a puncture hole found at the medial side. **C:** At 2 weeks, the elastic fibers of the IEL were torn apart from each other. The pattern was more prominent in the lower IVC segment (**E**) compared with the upper one (**D**). **F:** At 4 weeks, the IEL of the lower segment further degraded. **G:** The elastic fibers were sparse. **G:** At 8 weeks, the IEL could hardly be identified in same area of the lower segment with only thin and dispersed fragments of elastic fibers remaining. **H:** The IEL of small venous branch remained intact. Original magnifications: $\times 2000$ (**C**); $\times 4000$ (**A, B, D–H**).

and the elastic fibers became thinner and were sparse (Figure 4F). At 8 weeks, severe IEL degradation was observed in the lower segment, where the IEL structure could hardly be identified and only thin and dispersed pieces of elastic fibers remained (Figure 4G). The IEL of the small venous branch connected with the IVC remained intact (Figure 4H).

Ultrastructural Changes of IEL in IVC of ACF Rats

An electron microscopic study was performed to investigate the ultrastructural changes of the IEL at 8 weeks. In the sham-operated rats, the cross-sectional view of IVC showed that the IEL was composed of arrays of elastic fibers in cross section distributing between the luminal endothelial cells and medial SMCs (Figure 5A). In the

longitudinal view, the elastic fiber was shown to be of large length (Figure 5B). The ultrastructural findings were consistent with the general structure of IEL identified in confocal microscopic study. The densities of the fibers counted in the cross-sectional views were 6.0 ± 1.0 and 4.7 ± 1.1 /electron microscopic field in the upper and the lower segments, respectively. When compared, the density did not significantly differ between both segments ($P = 0.08$). In the upper IVC segment of ACF group, the ultrastructural pattern of IEL was similar to that of the sham-operated rats, but the elastic fibers were less densely distributed (Figure 5, C and D). In the lower IVC segment, cross-sectional view showed sparsely distributed elastic fibers (Figure 5E) of much smaller diameter compared with that of sham-operated rats. In the longitudinal view, fragments of elastic fibers (Figure 5F) of small diameter were found. Fragmentation of some elastic fibers was also found (Figure 5G). In some foci where large neointimal tissues were present, no elastic fibers could be found between the media and neointima (Figure 5H). In the ACF rats, the mean density of elastic fibers was lower in the lower IVC segment compared with the upper one (2.4 ± 1.0 versus 4.0 ± 1.4 , $P = 0.012$). The mean densities of the upper and lower IVC segments of the ACF rats were significantly lower compared with that of the corresponding segments of the sham-operated rats (Figure 5I). Similar changes were also observed for the diameters of elastic fibers between both groups (upper: $0.6 \pm 0.3 \mu\text{m}$ versus $1.5 \pm 0.5 \mu\text{m}$, $P < 0.001$; and lower: $0.5 \pm 0.3 \mu\text{m}$ versus $1.2 \pm 0.4 \mu\text{m}$, $P < 0.001$). The mean diameter was not different between the upper and the lower segments in the ACF group ($P = 0.592$) and was larger in the upper segment in the sham-operated group ($P = 0.006$) (Figure 5J). In addition to the ultrastructural changes of IEL, hyperplasia of the media that was composed of more layers of SMCs was also observed in the lower IVC segment of ACF rats (Figure 5, A and B). Consistent with the finding of the general histological study, neointimal tissues were found in the lower (Figure 5H) but not in the upper IVC segment of ACF rats.

Expression of Elastogenesis- and Elastinolysis-Associated Molecules

Balance between elastogenesis and elastinolysis is essential for homeostasis of IEL. Therefore, we investigated the expression profile of elastogenesis-associated molecules, elastases and, their inhibitors using RT-PCR at 2, 4, and 8 weeks after surgery ($n = 3$ for each time point in both groups). As shown in Figure 6, A and B, the mRNA levels of key elastogenesis-associated genes, including fibrillin-1, lysyl oxidase, and tropoelastin,¹⁹ did not differ between the sham-operated and the ACF groups at any time point (all $P > 0.05$ at each time point). The mRNA levels of elastases, including cathepsin S, cathepsin K, and MMP-2, were found to be up-regulated in the ACF group at any time point when compared with those in the sham-operated group ($P = 0.018$, $P = 0.005$, and $P = 0.001$ at 2 weeks; $P = 0.006$, $P = 0.004$, and $P = 0.003$

Figure 5. Representative electron micrographs of the IEL of IVC at 8 weeks. **A** and **B**: The IVC of sham-operated rats: The cross-sectional view showed that the IEL was composed of arrays of elastic fibers (**A**, arrowheads). The longitudinal view showed that the fibers were of long length (**B**, arrowheads). **C** and **D**: The upper IVC segment of the ACF rats. The ultrastructural pattern of the IEL (arrowheads) in cross-sectional (**C**) and longitudinal views (**D**) was similar to that of the sham-operated rats except that the elastic fibers were less densely distributed (**C**, arrowheads). **E** and **F**: The lower IVC segment of the ACF rats. Cross-sectional view showed sparsely distributed elastic fibers (**E**, arrowheads) of much smaller diameter. In the longitudinal view, fragments of elastic fibers (**F**, arrowheads) of small diameter were found. **G**: Fragmentation of the elastic fibers (arrows) was found in the lower IVC segment of ACF rats. **H**: Cross-sectional view of the IVC of ACF rats showed that no elastic fibers could be found between the media (M) and neointima (N). **I** and **J**: Comparison of the density (**I**) and diameter (**J**) of elastic fibers between the sham-operated and ACF rats. All except **G** were stained with uranyl acetate and lead citrate. **G** was stained with tannic acid-uranyl acetate followed by the lead citrate to enhance the staining of fragmented elastic fiber. Original magnifications: $\times 4000$ (**A–G**); $\times 2000$ (**H**).

at 4 weeks, and $P = 0.007$, $P = 0.017$, and $P = 0.005$ at 8 weeks). The highest expression levels of cathepsin K and cathepsin S were found to be at 2 and 4 weeks after surgery, respectively, whereas the expression level of MMP-2 remained constant at all time points. The mRNA levels of cystatin C, the most efficient endogenous inhibitor of cathepsins S and K, did not differ between the sham-operated and the ACF groups at any time point (all $P > 0.05$ at each time point). In contrast, the mRNA levels of TIMP-2, the endogenous inhibitor of MMP-2, were down-regulated in the ACF group at all time points ($P = 0.024$, $P = 0.012$, and $P = 0.016$ at 2, 4, and 8 weeks).

The expressions of cathepsin K, cathepsin S, and MMP-2 protein and their spatial association with the IEL were further investigated at 2 weeks after surgery ($n = 3$ in both groups) by immunofluorescent study. The specimens were observed by confocal microscope to detect the IEL and the immunostaining signals simultaneously. The results showed weak cathepsin S expression in IVC of the sham-operated group, which distributed evenly in the entire segment (Figure 7, A and B). In the IVC of ACF group, the expression of cathepsin S was increased compared with that of the sham-operated group (Figure 7,

C–F) with much stronger signals in the lower segment, especially in the intima (Figure 7, E and F). Immunostaining for the cathepsin K showed a similar pattern as for the cathepsin S (Figure 7, G–L) except that the increased expression of cathepsin K in the lower segment of ACF group distributed not only in the intima but also in the media (Figure 7, K and L). Immunostaining for MMP-2 was weak in the sham-operated group (Figure 7, M and N) and increased markedly at the media in the ACF group (Figure 7, O and P). To identify the cell types that express these elastases, double-labeling immunofluorescence was done using anti-CD31, anti-SM α -actin, and anti-CD3 as cell markers for endothelial cell, SMC, and T lymphocyte, respectively. Observation by confocal microscope showed that anti-cathepsin S was co-localized with anti-CD31 (Figure 8A), but not with anti-SM α -actin (Figure 8B) or anti-CD3 (data not shown) indicating that the cathepsin S was expressed in the endothelial cells. Anti-cathepsin K was also co-localized with the anti-CD31 (Figure 8C) and was co-localized with anti-SM α -actin in some foci of media (Figure 8D). It was not co-localized with anti-CD3 (data not shown). The findings indicated that cathepsin K was expressed mainly in the endothelial

Figure 6. A: Expression profile of mRNA of the elastogenesis-associated molecules, elastases, and inhibitors of elastases in IVC of sham-operated and ACF rats. **B:** Quantification of relative mRNA levels of cathepsin K, cathepsin S, and MMP-2. TIMP, tissue inhibitor of metalloproteinase.

cells and to a lesser extent in the medial SMCs. In contrast, the anti-MMP-2 signal was co-localized with anti-SM β -actin signals at media, but not with anti-CD31 or CD3 signals (Figure 8, E and F), indicating expression of MMP-2 in medial SMCs.

Activities of Cathepsin S, Cathepsin K, and MMP-2

Then we assessed the enzyme activities of cathepsin S, cathepsin K, and MMP-2 of IVC in both groups at 2 weeks after operation ($n = 3$ in both groups). A fluorescence-based assay showed significantly increased enzyme activities of cathepsin S and K of the lower IVC segment of ACF rats when compared with those of the sham-oper-

Figure 7. Representative immunoconfocal images of anti-cathepsin S, anti-cathepsin K, and anti-MMP-2 labeling in the IVC of sham-operated rats (**A**, **G**, and **M**) and the upper (**C** and **I**) and lower (**E**, **K**, and **O**) IVC segments of the rats with ACF at 2 weeks. **B**, **D**, **F**, **H**, **J**, **L**, **N**, and **P** are superimposed confocal images corresponding to **A**, **C**, **E**, **G**, **I**, **K**, **M**, and **O** to demonstrate the spatial correlation of the immunostaining signals (red) and the IEL detected by the autofluorescence of elastin (green) with cell nuclei stained with Hoechst stain (blue). Original magnifications, $\times 4000$.

ated rats ($P = 0.001$ and $P = 0.001$, respectively). In contrast, the activities of the upper IVC segment of ACF rats did not differ from that of the sham-operated ones ($P = 0.812$ and $P = 0.185$ for cathepsin S and cathepsin K, respectively). When compared between both segments of ACF rats, the activities of the lower segment were also significantly higher than that of the upper segment ($P = 0.004$ and $P = 0.002$ for cathepsin S and cathepsin K, respectively) (Figure 9A). Gelatin gel zymography was performed to assess the MMP-2 activity of IVC. Because the immunohistochemistry study showed homogenous expression of MMP-2 protein throughout the whole IVC segment in both groups, tissue lysate of

Figure 8. Representative double-labeling immunofluorescence images to identify the cell types that express the cathepsin S, cathepsin K, and MMP-2 (red color) in IVC of ACF rats at 2 weeks after operation. Specific cell markers, the CD31, and smooth muscle (SM) β -actin (blue color) were used to identify the endothelial cells and SMCs. **A and B:** Anti-cathepsin S was co-localized (purple to pink) with the anti-CD31, but not anti-SM β -actin, indicating expression of cathepsin S by endothelial cells. **C and D:** Anti-cathepsin K was co-localized (pink) with anti-CD31 and was also partially co-localized with anti-SM β -actin indicating expression of cathepsin K mainly by endothelial cells and to a lesser extent by SMCs. **E and F:** Anti-MMP-2 was co-localized with the anti-SM β -actin, but not anti-CD31, indicating expression of MMP-2 by SMCs. Original magnifications, $\times 6300$.

the whole IVC was submitted for assessment of MMP-2 activity. The MMP-2 activity of IVC in ACF rat was significantly higher than that of the sham-operated group ($P = 0.027$) (Figure 9B).

Discussion

This study demonstrates degradation of the IEL in vein graft of rat with ACF. When the vein graft was exposed to a hemodynamic condition of highly increased flow veloc-

ity and mildly increased intraluminal pressure, severe destruction of the IEL was found and complete loss of IEL structure was observed in some foci. The ultrastructural study revealed that the degradation process involved fragmentation and decrease in the density and diameter of the elastic fibers.

There are two potential mechanisms that may cause IEL degradation in this vein graft model. The first, progressive dilation of the IVC may stretch the IEL. The increased flow velocity and consequently increased flow volume would stretch the IVC immediately and cause positive remodeling later as demonstrated by the findings of increased circumference. Dilation of the IVC may pull apart the elastic fibers of the IEL and result in the low density of the elastic fibers in ACF rats. The second potential mechanism that may be involved in the IEL degradation in the ACF rats is the increased elastolytic activity. The homeostasis of the elastic fibers depends on the balance between elastogenic and elastolytic activities. In the present study, we found that the expressions of key elastogenesis-associated molecules in IVC did not differ between the ACF and sham-operated rats. In contrast, increased expression levels and enzyme activities of elastases including the cathepsins S and K and MMP-2 were demonstrated in the IVC of ACF rats. Cathepsins S and K have been shown to be potent elastases of the cysteine protease family in mammals.^{20,21} Cathepsin K is even the most potent among all of the elastases.^{22,23} The expressions of cathepsins K and S in IVC of ACF rats were localized by immunohistochemistry study predominantly to the luminal endothelial cells. The intimate spatial relationship between the luminal endothelial cells and IEL strongly supports that up-regulation of these potent elastases plays an important role in degradation of the IEL. The stronger immunostaining intensity of the cathepsins K and S in the lower IVC segment compared with that in the upper one is also consistent with the more severe IEL degradation in the lower segment. In cultured mouse aortic endothelial cells, different shear conditions have been shown to regulate the expressions of cathepsins.^{24,25} In the ACF rats, duplex scan demonstrated a high-velocity turbulent flow in IVC. These hemodynamic changes may be responsible for the up-regulation of cathepsins K and S. Up-regulation of the MMP-2 in the IVC of ACF rats may also contribute to the degradation of IEL.

In the present study, the neointimal hyperplasia developed predominantly at the lower IVC segment of ACF rats, where severe IEL degradation was found. In some foci where the IEL was disrupted, neointimal tissue was found to emerge through these defects. These findings suggest that the IEL degradation may contribute to the development of neointimal hyperplasia in the IVC of ACF rats. IEL destruction may not only indicate loss of the integrity of structural barrier, which may prompt medial SMCs to migrate to the intima. The elastin degradation peptides, products of degraded elastic fiber, have been shown to stimulate the proliferation of arterial SMCs²⁶ and are chemotactic to the monocytes,²⁷ both of which are important pathogenic mechanisms of neointimal hyperplasia.

Figure 9. Enzyme activities of elastases of IVC. **A:** Activities of cathepsin S and cathepsin K were assessed by fluorescence-based assays using the preferred cathepsin K or S substrate sequence. The relative activities of the upper and lower IVC segments of ACF rats and the IVC of sham-operated rats were compared. **B:** Activities of MMP-2 were assessed using gelatin zymography. The relative enzyme activities of the IVC of both groups were compared.

The vein graft model of ACF rats is hemodynamically similar to that of the arteriovenous fistula for hemodialysis access of uremic patients. The hemodialysis access is a vascular setting prone to development of neointimal hyperplasia,²⁸ which leads to outflow stenosis and consequently dysfunction of vascular access.²⁹ Degradation of the IEL found in the IVC of ACF rats may also occur in the venous limb of the hemodialysis access and contribute to the development of neointimal hyperplasia.

In summary, we show IEL degradation in vein grafts exposed to a flow of high velocity and a mildly increased pressure. Severe degeneration occurred in the segment exposed to prominent hemodynamic stresses, which may contribute to the development of neointimal hyperplasia in vein grafts.

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