Oxidative stress is involved in the development of experimental abdominal aortic aneurysm: A study of the transcription profile with complementary DNA microarray

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Background: **The role of oxidative stress in the formation of aneurysms is not fully understood. We used the complementary DNA (cDNA) microarray technique to determine the transcription profile in the development of elastase-induced abdominal aortic aneurysm in rat models, with an emphasis on the oxidative stress-related genes.**
Materials and Methods: **In the experimental group, rat abdominal aortas were perfused with elastase to induce AAA. In the control group, a sham operation was performed with perfusion of the aortas with saline solution. Four or five animals were used for each time point for each of the elastase-treated or saline-treated groups. At day 2, day 7, and day 10 after surgery, the external aortic diameter was measured and AAA formation was estimated. Total RNA was isolated from aortas and subjected to cDNA microarray analysis with the use of the rat genome U34A high-density oligonucleotide DNA chip (Affymetrix, Santa Clara, Calif), which contains a total number of 8799 genes of which 2017 are expressed sequence tag (EST) genes. The data were analyzed with the GENECHIP Data Mining Tool software (Affymetrix). For genes of interest, reverse-transcription polymerase chain reaction was performed to confirm their expression level.**
Results: **Comparison ranking analysis revealed that during AAA development, the expression of 212 genes, including 46 of EST genes, increased by more than two-fold and 229 genes, including 95 of EST genes, decreased by more than two-fold in at least one of the three time points. The regulated genes included those encoding heme oxygenase, inducible nitric oxide synthase, some extracellular matrix proteins, members of the matrix metalloproteinase family, and those associated with prooxidant/antioxidant and inflammatory responses. Reverse-transcription polymerase chain reaction analysis confirmed the upregulation of genes involved in oxidative stress, such as heme oxygenase, inducible nitric oxide synthase, 12-lipoxygenase, and heart cytochrome c oxydase subunit VIa, and the downregulation of antioxidant genes, such as superoxide dismutase, reduced nicotinamide adenine dinucleotide–cytochrome b-5 reductase, and glutathion S-transferase.**
Conclusion: **The cDNA microarray technique was useful for investigation of the transcription profiles during the development of AAA. Our results indicate that oxidative stress may play a pivotal role in the pathologic progression of AAA. (J Vasc Surg 2002;36:379-85.)**

Abdominal aortic aneurysm (AAA) represents a potentially fatal disorder that affects 2% to 9% of the population older than 65 years and remains an important clinical problem.[1] With the clinical features of a deteriorated aortic wall leading to progressive dilatation and eventual rupture, AAA results from atherosclerotic change followed by accelerated degradation of collagen and elastin, the main components of the extracellular matrix (ECM) in the vascular wall. In addition to these pathologic alterations, AAAs are associated with marked alterations of the cellular composition of the aortic wall, such as the infiltration of macrophages and T-lymphocytes into the adventitia and the greatly decreased population of vascular smooth muscle cells (VSMCs).[2,3]

To date, the most popular experimental model of AAA is the elastase-induced aneurysm in the rat model described by Anidjar et al.[4] In this model, the transient intraaortic infusion of porcine pancreatic elastase causes an inflammatory response in the aortic wall. With such experimental conditions, the infusion of elastase does not result in a rapid degradation of the elastic fibers, and generally only mild dilatation is seen immediately after elastase infusion, followed by a later increase in aortic diameter (AD) to aneurysmal proportions.[5,6] The associated pathologic events include the increased transmural infiltration of macrophage and T-lymphocytes, the production of matrix metalloproteinases (MMPs), such as MMP-2, MMP-9, and MMP-12, and the degradation of the medial elastic lamellae.[6-8] Al-

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though the elastase-induced AAA is not originated from atherosclerosis, the use of this model has been valuable for investigation of the pathogenesis of the disease because this model shares many pathologic features with human AAA.[5]

An increased body of evidence shows that oxidative stress caused by reactive oxygen species (ROS) participates in regulation of endothelium-dependent relaxation and modulation of VSMC phenotype and secretion of ECM. All of these events play important roles in vascular diseases, such as hypertension and atherosclerosis,[9] suggesting that the sources of ROS and the signaling pathways that they modify may be involved in development of AAA.

Many previous reports have revealed the gene expression in human AAAs or rat experimental AAAs. Most of these reports have concentrated on a few genes related to the pathologic events mentioned previously. The development of chip-based or membrane-based complementary DNA (cDNA) microarrays has made possible the investigation of the transcription profiles in cells in physiologic or pathophysiologic conditions.

This study uses the high-throughput cDNA microarray to determine the genes involved in the pathologic progression of aneurysmal formation. We found that genes related to oxidative stress, and those involved in inflammatory responses, are modulated in the experimental AAA.

MATERIALS AND METHODS

Aneurysm formation. The procedures used in this study were according to those reported by Yamaguchi et al,[10,11] with slight modifications, and were approved by the University of California–Riverside Chancellor's Committee on Laboratory Animal Care. Male Sprague-Dawley rats (approximately 300 g) were anesthetized with intraperitoneal injection of 6% sodium pentobarbital (50 mg/kg). The infrarenal abdominal aorta was dissected from the inferior vena cava and other surrounding tissues, and all lumbar arteries within this portion were ligated. A polyethylene PE10 catheter (Baxter, Mcgraw Park, Ill) was inserted through the left femoral artery into the aorta until the tip was just about to reach the inferior mesenteric artery. A ligature was placed around the catheter and the surrounding aorta, and an aortic clamp was placed at the distal level of the left renal vein. The isolated segment (approximately 1 cm) then was perfused continuously with a syringe pump for 30 minutes; the experimental group received 1 mL of saline solution containing 15 U/mL of porcine pancreatic elastase type 1 (Sigma, St Louis, Mo), and the control group received only 1 mL of saline solution.[10,11] After the perfusion, the left femoral artery was ligated at the proximal point of the insertion. The wounds were closed, and the animals were allowed to recover from anesthesia. On postoperational day (POD) 2, POD7, and POD10, aortas were resected. The external AD was measured with a digimatic caliper with the microscope before and after perfusion and at harvest. The resected aortas were washed with cold phosphate-buffered saline solution to remove blood and were preserved in liquid nitrogen until use. Representative sections from each specimen were subjected to Verhoeff–van Gieson staining and hematoxylin and eosin staining. The degrees of degradation of elastin and inflammation in the aneurysm models were assessed with comparison with the controls.

RNA extraction, array hybridization, scanning, and data analysis. Total RNA was isolated from the resected aortas with TRIzol (Life Technology, Grand Island, NY). Equal amounts of RNA isolated from different animals within the same subgroup were pooled together. cDNA was synthesized with an oligo-dT primer attached to a sequence of the T7 promoter region. cRNA then was generated with an ENZO biotinylated labeling kit (ENZO Biochem, New York, NY), followed by fragmentation to an average size of 100 to 150 bases, which then were hybridized with a rat genome U34A array (Affymetrix) at 45° C for 16 hours. The hybridized arrays were washed, stained, and scanned according to standard protocols. The results were analyzed with the GENECHIP analysis suite software (Affymetrix).

Each pooled RNA sample was subjected to two chip hybridization, and each individual microarray scanning result was analyzed to determine the expression levels of individual genes. Comparison ranking analysis was made on the results among two samples for the experimental group and two for the controls for each of the three time points. The detailed procedure of the analysis is described in the online supplement.

Reverse-transcription polymerase chain reaction. For confirmation of the results of microarray analysis, reverse-transcription polymerase chain reaction (RT-PCR) was performed on seven oxidative stress-related genes with the Reverse Transcription System (Promega, Madison, Wis). The RT reaction with 0.2 μg of RNA from the same pooled samples was performed with the following cycles: 42° C for 60 minutes, 95° C for 5 minutes, and then cooling to 4° C. For each reaction, cDNA from the RT reaction was amplified with PCR with the use of 2.5 units of *Taq* DNA polymerase (Promega). The PCR cycles for each reaction were as follows: heat denaturation at 94° C for 1 minute, primer annealing at 55° C for 2 minutes, and primer extension at 72° C for 3 minutes. Table I (online only) summarizes the primer sequences of various genes analyzed and the number of cycles of PCR reaction. The PCR reactions were carried out in a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, Calif). Aliquots of each PCR reaction were separated with 2% agarose gels and revealed with ethidium bromide under ultraviolet light. The gel was photographed, and the density of DNA bands was analyzed with National Institutes of Health image software.

Statistics. The preperfusion, postperfusion and final AD measurements for the experimental and control groups were recorded as mean ± standard error, and the data were analyzed with the Student *t* test. For each animal, the *percentage increase of AD* was defined as (AD final − AD preperfusion)/AD preperfusion × 100%. The difference was considered significant at a P value of less than .05. Statistical analysis for cDNA microarray result was per-

Table II. AD measurements in elastase-perfused or saline-perfused aortas

Group	*AD before (mm)*	*AD after (mm)*	*AD final (mm)*	*AD increase (%)*
C-2 (n = 4)	1.37 ± 0.06	1.78 ± 0.02	1.68 ± 0.02	22.6 ± 4.0
E-2 (n = 4)	1.28 ± 0.05	1.72 ± 0.11	2.58 ± 0.14*	102 ± 7.6*
C-7 (n = 5)	1.36 ± 0.08	1.85 ± 0.19	1.62 ± 0.13	19.1 ± 7.4
E-7 (n = 4)	1.31 ± 0.02	1.80 ± 0.02	6.54 ± 0.71*	398 ± 57*
C-10 (n = 4)	1.39 ± 0.07	1.75 ± 0.05	1.56 ± 0.06	12.3 ± 1.7
E-10 (n = 4)	1.36 ± 0.08	1.80 ± 0.09	9.36 ± 1.24*	589 ± 58*

Numbers in parentheses indicate days after operation. *AD before* and *AD after* diameters measured before perfusion or 5 minutes after blood restoration, whereas *AD final* diameters at resection.
*$P < .05$ when compared with parallel saline-perfused controls and with other two elastase-treated groups.
C, Saline-perfused controls, *E*, elastase-treated experimental group.

formed with comparison ranking within software Data Mining Tool (version 2.0, Affymetrix).

RESULTS

Aneurysm formation. The mean AD of rats increased by 30% to 35% immediately after aortic perfusion with either elastase or saline solution (Table II). In saline-perfused controls, the AD subsequently decreased to within the normal range. In elastase-perfused animals, the AD was slightly enlarged at POD2 but increased more markedly thereafter; aneurysmal formation was observed at POD7, and further enlargement occurred at POD10 in all animals. The histologic features of these specimens were consistent with previous reports.[6-8]

Complementary DNA microarray analysis. Depending on the change of fluorescence intensity, the 8799 probe sets in the chip were divided into five groups according to the manufacturer's software: 1, increase (I); 2, marginal increase (MI); 3, no change (NC); 4, marginal decrease (MD); and 5, decrease (D). From four comparisons between the elastase-treated and the corresponding saline-treated groups (two chips each), the genes whose expression level was either (I) or (D) in both chips were denoted as being up-regulated or down-regulated, respectively. The degree of change for each gene in the (I) or (D) groups then was averaged from the two paired measurements, and only those with a change greater than 2.0 or smaller than −2.0 were selected.

After comparison ranking analysis, up-regulated or down-regulated genes were selected from those encoding for known genes. At POD2, POD7, and POD10, the numbers of up-regulated genes were 97, 68, and 79, respectively, and those of down-regulated genes were 65, 84, and 60, respectively. Fourteen genes were induced and 15 were suppressed at both POD2 and POD7, 18 genes were induced and five suppressed at both POD2 and POD10, and 18 genes were induced and 15 suppressed at both POD7 and POD10. Fourteen genes were consistently induced and 20 were consistently suppressed during the course of AAA, namely, at POD2, POD7, and POD10 (Tables III and IV, online only).

Among the up-regulated or down-regulated genes, those related to vascular biology or immune/inflammation conditions are listed in Tables V and VI. These included 28 increased and 15 decreased expressions in the elastase-induced rat AAA. The genes whose expressions increased at all these time points were interleukin-6, plasminogen activator inhibitor–1, and urokinase type plasminogen activator receptor–1. Inducible nitric oxide synthase (iNOS), interleukin-1β, macrophage inflammatory protein–2 precursor, DORA protein, and collagenase were up-regulated at both POD2 and POD7. The genes in which expression increased at both POD7 and POD10 include heme oxygenase (HO) and heart cytochrome c oxidase subunit VIa. The expression of 12-lipoxygenase and CXC chemokine receptor increased at both POD2 and POD10. Among suppressed genes, down-regulated at all time points were superoxide dismutase (SOD), epididymal secretory superoxide dismutase, reduced nicotinamide adenine dinucleotide (NADH)–cytochrome b-5 reductase, glutathione S-transferase (GST), tropoelastin, SC1 extracellular matrix glycoprotein, SM22, myosin regulatory light chain isoform C, and alternatively spliced smooth muscle myosin heavy chain.

At POD2, POD7, and POD10, the total numbers of up-regulated expressed sequence tag (EST) genes were 7, 16, and 8, and those of down-regulated EST genes were 34, 18, and 10, respectively. Three genes were commonly induced and six were suppressed at both POD2 and POD7, two genes were induced and eight were suppressed at both POD2 and POD10, and five genes were induced and 11 were suppressed at both POD7 and POD10. Five genes were consistently induced and eight were consistently suppressed at POD2, POD7, and POD10. Table VII (online only) shows the gene list of Affymetrix Rat genome U34A chip, and the tables of up-regulated and down-regulated EST genes are in Tables VIII and IX (online only).

Reverse-transcription polymerase chain reaction confirmation. The results from cDNA microarray analysis were confirmed with RT-PCR for seven genes related to oxidative stress. At POD2 and POD7, the iNOS messenger RNA (mRNA) level in the elastase-treated specimens was greater than that in controls perfused with saline solution. The level of HO mRNA was increased at all time points. The expression of the 12-lipoxygenase gene was up-regulated in the elastase group at POD2 and POD10, and that of the cytochrome b558 α-subunit genes was induced at POD2. The RT-PCR results for SOD, NADH-cytochrome

Table V. Up-regulated genes during AAA formation and their fold of change

Gene (Genbank No.)	*POD2*	*POD7*	*POD10*
Oxidative stress			
iNOS (U03699)	52	5.2	NC
iNOS2 (U48829)	10	NC	NC
iNOS (D44591)	27	NC	NC
Argininosuccinate synthetase (X12459)	5.6	NC	NC
Heme oxygenase (J02722)	NC	3	10
12-Lipoxygenase (S69383)	4	NC	2.6
Cytochrome b558 α-subunit (U18729)	2.6	NC	NC
Oxidative stress-inducible protein tyrosine phosphatase (S81478)	4.3	NC	NC
5-Lipoxygenase activating protein (X52196)	3.2	NC	NC
Heart cytochrome c oxidase subunit VIa (X12554)	NC	3.9	2.7
Inflammation			
Interleukin-6 (M26744)	13	15	5.7
Interleukin-1α (D00403)	8.4	NC	NC
Interleukin-1β (E01884)	37	5.5	NC
Interleukin-1β (M98820)	23	6	NC
Interleukin-1 receptor type 2 (Z22812)	10	9.1	NC
CXC chemokine receptor (U90610)	3.7	NC	4.2
CC chemokine receptor protein (E13732)	5	NC	NC
CXC chemokine *LIX* (U90448)	NC	8.9	NC
Tumor necrosis factor I receptor (AA900380)	8.5	NC	NC
Macrophage inflammatory protein-1α (U22414)	39	NC	NC
Macrophage inflammatory protein-2 precursor (U45965)	40	5.5	NC
DORA protein (AJ223184)	5.4	3.9	NC
Fibrinolysis			
Plasminogen activator inhibitor-1 (M24067)	2.4	7.1	3
Urinary plasminogen activator receptor (X71898)	13.6	3.5	9.8
MMP family			
Gelatinase B (MMP-9) (U24441)	5.9	NC	NC
Macrophage metalloelastase (MMP-12) (X98517)	5.3	–5.5*	–2.7*
Neutrophil collagenase (MMP-8) (AJ007288)	3.6	NC	NC
Collagenase (M60616)	4.8	12.2	NC

*Gene expression was decreased at POD7 and 10.
NC, No change.

b5 reductase, and GST showed a reduced mRNA level in the experimental group compared with controls at each time point. As shown in Fig, all the PCR results agreed well with those of the microarray analysis (Tables V and VI), except that the time course of the change in the HO mRNA was reversed between the RT-PCR and the microarray analysis. Serving as a control, glyceraldehyde-phosphate dehydrogenase mRNA showed similar levels in all experiments, which not only indicated that the reduced glyceraldehyde-phosphate dehydrogenase gene was not affected during AAA but also validated the RT-PCR experimental procedures.

DISCUSSION

The use of elastase-induced AAA in the rat model has facilitated the investigation of transcription regulation during the onset of AAA. Genes encoding for the MMP family, ECM proteins, cytokines, cycloxygenase-2, and iNOS have been reported to be up-regulated in rat AAAs.[12,13] Most of these reports focused on a small number of genes, and information is lacking on the temporal responses of these genes. The advancement of cDNA microarray has allowed a high-throughput survey of gene expression during the pathogenesis of many diseases, including atherosclerosis and aneurysm.[14,15] Recently, a microarray study investigated the expression profile of human AAA compared with specimens from young organ transplant donors.[15] We compared the data generated from this study and those from the previous human AAAs. Only a few genes (eg, gelatinase B and CXC chemokine receptor) are commonly modulated in our rat models and in human AAA. One possible explanation is that our samples were harvested during the process of AAA formation, whereas those in the study of Tung, Lee, and Thompson[15] can be regarded as "completed" AAAs. Thus, gene expression profile may be dependent on distinct periods during development of AAA.

Oxidative stress contributes to various cardiovascular diseases,[9] but little information is available on the role of oxidative stress-related genes in AAA. In this study, we showed that the iNOS and HO genes are induced at POD2 and POD7 and at POD7 and POD10, respectively. NO, modulated by iNOS, plays a pivotal role in cardiovascular homeostasis, atherosclerosis, and inflammatory responses. One of the mechanisms with which nitric oxide (NO) mediates tissue damage is the enhancement or amplification of the effects of proinflammatory cytokines on various cell types, which in turn may increase the expression and enzymatic activity of MMPs.[16,17] In addition, NO can

Table VI. Down-regulated genes during AAA formation and fold of change

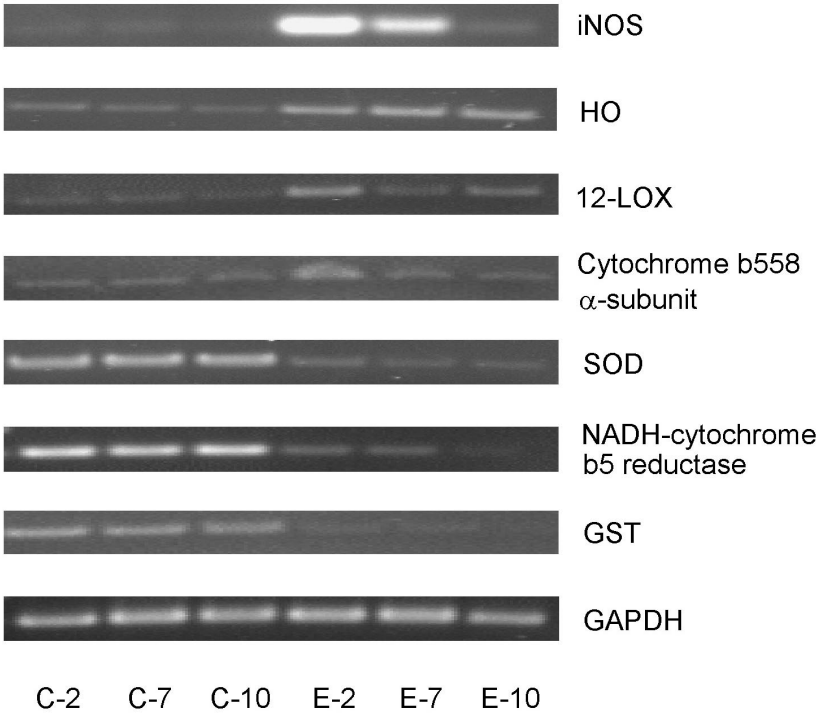
No. gene (Genbank)	*POD2*	*POD7*	*POD10*
Antioxidative stress			
SOD (Z24721)	−6.4	−8.9	−21
Epididymal secretory superoxide dismutase (X68041)	−8.9	−5.6	−12
NADH-cytochrome b-5 reductase (J03867)	−2.7	−2.3	−2.8
NADH-cytochrome b-5 reductase (D00636)	−2.5	−6.6	−11
GST (J03752)	−2.2	−3.2	−2.8
ECM			
Tropoelastin (J04035)	−2.4	−3.3	−4.2
*SC*1 protein (U27562)	−68	−19	−61
Tenascin-X (U24489)	NC	−6.9	−9.6
Fibromodulin (X82152)	−2.9	NC	−2
TSP-4 protein (X89963)	−4.6	NC	NC
Cytoskeleton			
*SM*22 (M83107)	−4	−3.6	−2.4
Myosin regulatory light chain isoform C (S77900)	−3.4	−2.3	−3.5
Alternatively spliced *SM* myosin heavy chain (X16262)	−5	−4.1	−6.3
α-Actinin-2 associated *LIM* protein (AF002281)	−3.5	−5	NC
Smooth muscle cell *LIM* protein (SmLIM) (U44948)	−2.5	NC	NC

NC, No change.

cause tissue damage through the nitration of ECM proteins in vivo[18] and can induce Fas-mediated VSMC apoptosis in vitro.[19] The induction of the iNOS gene is consistent with the previous observation that a selective blocking NO production with aminoguanidine reduces the aneurysm size in elastase-induced rat AAAs.[13] However, AAA is still induced with elastase in the iNOS-deficient mice.[16] Thus, the role of NO and iNOS in this model remains to be elucidated. Another diatomic gas molecule involved in tissue damage is carbon monoxide (CO), which is produced as a byproduct of heme catabolism catalyzed by HO. Through a cyclic guanosine monophosphate–dependent mechanism, CO is similar to NO in causing blood vessel relaxation and platelet inhibition.[20,21] Furthermore, NO can enhance HO activity to generate biliverdin, which is subsequently converted to bilirubin with biliverdin reductase.[22] Both biliverdin and bilirubin are efficient scavengers of ROS and hence lead to cellular defense mechanisms against oxidative tissue injury.[23,24] The temporal responses of iNOS and HO indicate that, although the generated NO and CO may be important in initiating tissue injury, the induction of HO may also exert protective effects during the AAA formation.

Several other genes related to ROS production were found to be modulated in our study. The level of cytochrome b558 α-subunit, a subunit of NADH oxidase, was increased at POD2. NADH oxidase is the main enzyme generating ROS in vascular tissues.[25] The level of 12-lipoxygenase, which causes intracellular membrane degeneration,[25] was increased at POD2 and POD10. However, 12-lipoxygenase was down-regulated at POD7, and this was confirmed with RT-PCR. The reason for this biphasic modulation of 12-lipoxygenase during AAA formation is unknown. Interestingly, the expression of several genes encoding for antioxidants, such as the SOD, NADH-cytochrome b5 reductase, and GST genes, was decreased throughout the experimental period. The distinct expression profiles of prooxidant and antioxidant genes would result in an imbalance between ROS production and free radical scavenge. As a result of the impaired redox state, oxidative damage may accelerate the formation of AAA. This notion is supported with the observation that an imbalance between prooxidants and antioxidants induces VSMC apoptosis after an acute vascular injury.[26] Although we were not able to detect the increase in the apoptosis-related genes, such as Fas or caspase family members, the down-regulation of genes encoding for VSMC cytoskeletal proteins, such as SM-22, and those encoding ECM proteins, such as tropoelastin, can be related to a reduction of VSMCs during AAA formation. Taken together, our results may imply that oxidative stress is induced during the process of AAA, which leads to tissue injury and VSMC apoptosis.

A number of genes involved in the inflammatory response, such as cytokines and their receptors, lymphocyte cell surface antigens, and immunoglobulin, were induced. Some of these genes, for example, interleukin-6 or interleukin-1β, are known to be involved in human or experimental AAAs.[27-29] The mRNA levels of interleukin-6, interleukin-1β, and tumor necrosis factor–α are up-regulated by ROS in a dose-dependent manner.[30] Thus, AAA formation can be modulated by ROS in many ways. Apparently, other proinflammatory genes shown in Table V also correlate with the pathogenesis of AAA. These genes and the MMP family members had their highest expression at POD2, just before the onset of the large increase in AD, which suggests that, like MMPs, these proinflammatory-related proteins play an important role in promoting AAA. We showed that the expression of MMP-12 was increased at POD2 but decreased at POD7 and POD10. In addition, the expression of all other MMPs shown in Table V decreased at POD10 to the levels of the sham controls. The



RT-PCR analysis of expression of seven genes related to oxidative stress during AAA formation in rat models. Total RNA isolated from various specimens was subjected to RT-PCR, and products were separated with agarose gel electrophoresis and visualized with ethidium bromide staining. Shown at *bottom* are sources of samples. *C* and *E* denote saline-perfused controls or elastase-treated experimental group, and *hyphenated numbers* represent numbers of days after operation. Reduced glyceraldehyde-phosphate dehydrogenase, which was used as internal control, shows uniform expression level.

mechanism underlying a temporal decrease in the gene expression of some MMPs is of interest. The link between changes in oxidative stress and the altered activities of the secreted MMPs deserves further study.

In this study, the limited availability of RNA from the rat aortas, especially in the controls, led to the combination of samples from different rats, thus imposing a statistical limitation in analyzing the microarray results. In microarray screening of gene expression, validation of the results with methods such as Northern blotting or RT-PCR is necessary. With the use of RT-PCR, we confirmed the microarray analysis of expression levels of oxidative stress-related genes during AAA development. Future studies, with ROS inhibitors or gene-deleted animal models, will be needed to establish the precise roles of genes in the pathogenesis of AAA. In addition, investigations of the roles of the unidentified ESTs in this disease also will be fruitful.

In summary, this study provides evidence of the utility of a genome-wide cDNA microarray screening to identify candidate genes involved in the etiology of AAA. Our data suggest that oxidative stress-imposed injury may be one of the key events leading to AAA formation. The role of ROS in inducing multiple pathways to promote AAA formation can be studied further with the use of ROS inhibitors to attenuate the AAA size.

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