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Expression of MMP-2, MMP-9 and TIMP-1 in the Wall of Abdominal Aortic Aneurysms

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An impaired mechanism of regulatory feedback by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) has been implicated in the development of abdominal aortic aneurysms (AAAs). This study examined the pathogenesis of AAAs with respect to pathological characteristics and expressions of MMP-2, MMP-9 and TIMP-1. Their expressions were evaluated by immunohistochemistry, competitive polymerase chain reaction (PCR) and Western blotting in a total of 23 consecutive AAAs. The AAA specimens were obtained by surgery, while control specimens were obtained at autopsy. Specimens consisted of 6 patients with small-diameter AAAs (30–45 mm), 17 with medium-large-diameter AAAs (> 45 mm) and 11 controls (17–25 mm). Immunohistochemistry showed MMP-2- and TIMP-1-positive cells mainly in the intima, and MMP-9-positive cells in the intima and adventitia. Competitive PCR showed a significantly higher expression of MMP-2 messenger RNA (mRNA) in the small-diameter AAAs, and higher expressions of MMP-9 mRNA in the small-diameter and medium-large-diameter AAAs than in the controls. The mRNA levels significantly correlated between TIMP-1 and MMP-9, and between MMP-2 and MMP-9 in the AAAs, especially in the medium-large-diameter AAAs. Western blotting revealed the expression of MMPs and TIMP-1 variably in all the specimens examined. These results indicated that MMP-2 and MMP-9 might act cooperatively and play a crucial role in the development of AAAs, and that TIMP-1 inhibits MMP-9 in the AAAs, especially in those medium-large-diameter AAAs.

Key words: abdominal aortic aneurysm; competitive polymerase chain reaction; immunohistochemistry; matrix metalloproteinase; tissue inhibitor of metalloproteinase

The pathologic hallmark of aortic aneurysm is considered to be the remodeling of the aortic wall, involving fragmentation and decrease of elastic fibers in the tunica media (Baxter et al., 1992; Gandhi et al., 1994). True aneurysms formed in Marfan's syndrome (Chikumi et al., 2000) were found to be due to a mutation in the fibrillin gene (Dietz et al., 1991; Tsipouras et al., 1992), indicating that aneurysms may be caused by genetic disorders affecting 2 or more components of the aortic wall. Recent hypotheses have indicated that matrix metalloproteinases (MMPs) involved in the metabolism of elastin in the wall of aortic aneurysms may be one of the etiologic factors in the development of abdominal aortic aneurysm (AAA) (Campa et al., 1987; Tilson et al., 1988; Baxter et al., 1992; Freestone et al., 1995; Newman et al., 1994; Thompson et al., 1995; McMillan et al., 1995, 1995, 1997; Tamarina et al., 1997; Davis et al., 1998; Elmore et al., 1998). However, the precise roles of monocytes, macrophages and vascular smooth muscle cells which are considered to be involved in the early changes

Abbreviations: AAA, abdominal aortic aneurysm; cDNA, complementary DNA ESDNA, external standard DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase;mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SAB, streptavidin-biotin-peroxidase; SCDNA, specimen cDNA;TIMP, tissue inhibitor of metalloproteinase

of vascular remodeling, have not been adequately elucidated. It is important to examine the entire wall as a functioning organ using molecular-biological techniques, to compare the pathological changes of the individual layers including the intima, tunica media and adventitia. AAAs might result from impairment of the mechanism of regulatory feedback by MMPs and tissue inhibitors of metalloproteinases (TIMPs). A comparative analysis of anatomic changes and tissue metabolism in AAAs small in diameter without obvious structural destruction may provide a clue to the pathogenesis.

With this background, the present study examined the metabolism of elastin in the human aortic wall during the process of expansion of aortic diameter with respect to the expression of MMP-2, MMP-9 and TIMP-1.

Materials and Methods

Specimens

The subjects in this study consisted of 23 consecutive patients with AAA who had undergone an elective excision with graft replacement between November 1998 and December 1999. Eleven specimens obtained at autopsy performed between February 1999 and March 2000 served as controls. Table 1 lists clinicopathological profiles of the AAA patients and the autopsy cases.

Measurement of aortic diameter and staging of AAA

During surgery, the maximal diameter of the aorta distal to the renal artery was measured directly using the divider. The aortic diameter in the control group was measured at autopsy. In this study, an aorta with a diameter more than 30 mm was defined as an AAA. An aorta with a diameter between 30 mm and 45 mm was defined as a small-diameter AAA, and that with a diameter over 45 mm as a medium-large-diameter AAA.

Method for collection of aortic wall

After approval by the Ethics Committee of Tottori University Faculty of Medicine, surgical specimens of the aortic wall were collected from the patients after obtaining informed consent. During surgery, a rectangular strip of the anterior wall was resected from the site of maximal diameter to the aneurysmal-normal junction throughout the wall. At autopsy, a section was obtained from the abdominal aorta with a grossly atheroma-free intima throughout the wall. Immediately after resection, part of each specimen was fixed in 10% neutral buffered formalin for 48 h, and the rest was frozen in liquid nitrogen and stored at –80˚C.

Histopathologic analysis

Three-micron-thick sections were cut from paraffin-embedded human AAA walls and abdominal aortas from autopsy materials. The sections were stained with hematoxylin-eosin staining and elastica van Gieson. For the localization of MMP-2, MMP-9 and TIMP-1, the streptavidin-biotin-peroxidase complex (SAB) method was used for immunohistochemical staining. As the first antibody, purified mouse monoclonal anti-human MMP-2 antibody (diluted 1:200), purified mouse monoclonal anti-human MMP-9 antibody (diluted 1:400), purified mouse monoclonal anti-human TIMP-1 antibody (diluted 1:200) (Fuji Chemical Inc., Takaoka, Japan), and purified mouse monoclonal anti-human HAM56 antibody (DAKO, Carpenteria, CA) were used. As the 2nd antibody and enzyme reagents, a Histofine SAB-PO kit (Nichirei, Tokyo, Japan) was used. Endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide at room temperature for 30 min. For the retrieval of the antigens, all slides were placed in 10 mmol/L citrate buffer (pH 6.0) and heated at 94˚C for 15 min in a microwave oven. Nonspecific staining was prevented by treatment with phosphate-buffered saline (PBS) containing 2% fetal calf serum. The sections were treated with diaminobezidine for color develop-

Table 1. Patient Characteristics

Variable	AAA	Small AAA†	Medium-large AAA‡	Control	*P* value
Number of patients					
Total	23	6	17	11	
Male	22	5	17	7	
Female	1	1	0	4	
Age (year)					
Mean ± SD	72.4 ± 6.8	70.3 ± 3.7	73.1 ± 7.8	66.1 ± 14.1	NS
Range	62–85	66–77	62–85	47–92	
Diameter (mm)					
Mean ± SD	53.3 ± 10.8	40.8 ± 5.6	57.5 ± 8.6	20.4 ± 2.4	< 0.01
Range	30–80	30–45	48–80	17–25	
History of smoking	16 (69.6)	5 (83.3)	11 (64.7)	7 (63.6)	NS
Chronic obstructive pulmonary disease	8 (34.8)	1 (16.7)	7 (41.1)	1 (9.1)	NS
Hypertension	14 (60.9)	4 (66.7)	10 (58.8)	2 (18.2)	NS
Diabetes mellitus	5 (21.7)	2 (33.3)	4 (23.5)	2 (18.2)	NS
Hyperlipidemia	6 (26.1)	2 (33.3)	4 (23.5)	2 (18.2)	NS
Ischemic heart disease	10 (43.5)	1 (16.7)	9 (52.9)	0 (0)	< 0.01
Arteriosclerosis obliterans	7 (30.4)	2 (22.2)	5 (29.4)	1 (9.1)	NS
Cerebrovbascular disease	3 (13.0)	1 (16.7)	2 (11.8)	3 (27.3)	NS
Malignant tumor	5 (21.7)	3 (50.0)	2 (11.8)	8 (72.7)	< 0.05

( ), %.
AAA, abdominal aorataic aneurysm; NS, not significant.
† Aneurysmal diameter, 30–45 mm.
‡ Aneurysmal diameter, > 45 mm.

ment, and counterstained with 3% methyl green. The degree of tissue staining was judged under a light microscope at 4 × 3.3 magnification for elastica van Gieson staining and at 20 × 5 magnification for immunohistochemical staining, and then the expression was analyzed.

Competitive polymerase chain reaction (PCR)

The frozen tissue samples were homogenized in guanidium isothiocyanate using a Polytron homogenizer (Kinematica, Luzern, Switzerland). After homogenization, total RNA was isolated by CsCl in ultracentrifugation at 100,000 *g* for 20 h. Total RNA was further treated with gribonuclease-free deoxyribonuclease I (Nippon gene, Tokyo) and extracted with phenol/chloroform/isoamylalcohol (50:49:1) and precipitated with ethanol. Reverse transcription of 1.5-μg total RNA was performed to synthesize complementary DNA (cDNA) using 2 μL of 500-μg/mL Random Hexamer (Promega, Madison, WI) and 2 μL of 200-units/μL Moloney murine leukemia virus reverse transcriptase (Gibco BRL Products, Life Technologies, Rockville, MD). Control reactions omitting the reverse transcriptase (reverse transcriptase negative) were set up for each RNA sample.

The primers of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MMP-2, MMP-9 and TIMP-1 were designed based on Tamarina's report (Tamarina et al., 1997) by using the Genetyx-Mac Macintosh software (Software Development Co., Ltd., Tokyo) (Table 2). We designed the external standard DNA (ESDNA) for each studied gene that lacked from 10 to 20 base pairs (bp) so that the ESDNA differed in size from the specimen cDNA (SCDNA) obtained by the previous method (Ho et al., 1989). The identity of the SCDNA and ESDNA was verified by DNA sequence analysis with ABI310 (Applied Biosystems, Tokyo).

The PCR reaction mixture (12 μL final volume) contained 0.64 μL of each cDNA, 0.5

Table 2. Primers used for competitive PCR

Gene	PCR	Length (bp)	Primer
MMP-2	SP	398	5′=CAGGCTCTTCTCCTTTCACAAC
			3′=AAGCCACGGCTTGGTTTTCCTC
	ESP	379	5′=ACTCCAGACCCCTGGCTTTT
			3′=CCAGGGGTCTGGAGTTGTCCCACTGCCCTGTGCCA
MMP-9	SP	172	5′=TGGGCTACGTGACCTATGACAT
			3′=GCCCAGCCCACCTCCACTCCTC
	ESP	162	5′=AAGGAGCCAGTTTGCCGGAT
			3′=GCAAACTGGCTCCTTGGTCCCAGTGGGGATTTACA
TIMP-1	SP	279	5′=GGGGCTTCACCAAGACCTACAC
			3′=AAGAAAGATGGGAGTGGGAACA
	ESP	267	5′=TCTGAAAAGGGCTTCCAGTC
			3′=GAAGCCCTTTTCAGACTGGTCCGTCCACAAGCAAT
GAPDH	SP	216	5′=TGCCTCCTGCACCACCAACTGC
			3′=ATGACCTTGCCCACAGCCTT
	ESP	205	5′=TGGAAGGACTCATGACCACA
			3′=TCATGAGTCCTTCCATTGTCATGGATGACCTTGGC

bp, base pair; ESP, external standard primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PCR, polymerase chain reaction; SP, specimen primer; TIMP, tissue inhibitor of metalloproteinase.

units Taq polymerase (Gene Amp Taq Gold, Perkin Elmer, Branchburg, NJ), 12 pmol of forward primer, 6 pmol of reverse primer conjugated with Cy5 (Amersham Pharmacia Biotech, Buckinghamshire, England) and 6 pmol of reverse primer. For each sample, 4 or 5 equal aliquots were prepared with a dilution series of the ESDNA, and the PCR mixture was spiked into these aliquots. Amplification was performed in the thermal cycler (Labosystems Japan Co., Tokyo) for 35 cycles: denaturation at 95˚C for 1 min; annealing for 1 min at 55˚C for GAPDH, MMP-9 and TIMP-1 and at 60˚C for MMP-2; and extension at 72˚C for 1 min.

As the reverse primer was fluorescently labeled with Cy5, PCR products were detected with an ALF red DNA Sequencer (Amersham Pharmacia Biotech). After competitive PCR was completed, the products corresponding to the SCDNA and the ESDNA were electrophoresed on a 6% polyacrylamide and 6 mol/L urea gel on the Sequencer. The peaks were detected and analyzed with the Allele Links software (Amersham Pharmacia Biotech). The specific SCDNA template in each starting cDNA sample was equimolar to ESDNA when that ESDNA area/SCDNA area was equal to 1 (Fig. 1). The RNA amount is presented as a percentage to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting

Frozen specimens of human AAA walls or autopsied abdominal aortic walls were homogenized in a homogenizer, and the homogenate was dissolved in lysis buffer containing 0.1% nonylphenoxy polyethoxy ethanol (NP-40). The solution was centrifuged in an ultracentrifuge at 15,000 revolutions per minute for 10 min, and the supernatant was used as a test sample. The concentration of protein was measured with a Protein Assay Kit (Bio-Rad, Hercules, CA). Test samples of the same protein content (375 μg) were electrophoresed on a 5% sodium dodecyl-sulfate-polyacrylamide gel at a constant voltage of 150 V, and the proteins were transferred onto a nitrocellulose membrane at 80 V for 150 min. Markers (Bio-Rad Labs., Richmond, CA) were dissolved in a Coomassie brilliant blue solution and used for electrophoresis. After blocking nonspecific reaction with 10% skim milk in PBS, the membrane was reacted at 4˚C overnight with one of the following first antibodies: purified mouse monoclonal anti-human MMP-2 antibody (diluted 1:50),

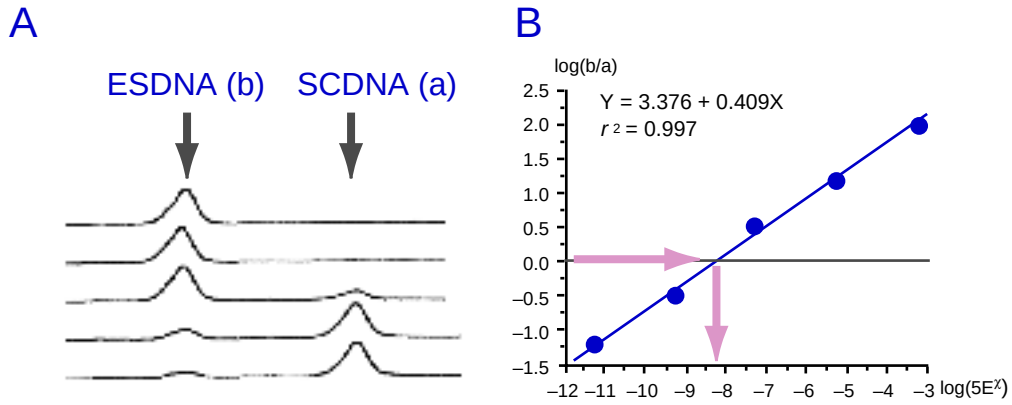

Fig. 1. **A:** Polymerase chain reaction (PCR) products were detected with an ALF red DNA Sequencer (Amersham Pharmacia Biotech), as the reverse primer was fluorescently labeled with Cy5. The Peaks were detected and analyzed with the Allele Links software (Amersham Pharmacia Biotech). **B:** The specific specimen cDNA (SCDNA) template in each starting cDNA sample was equimolar to the external standard DNA (ESDNA) when that ESDNA area/SCDNA area was equal to 1.

purified mouse monoclonal anti-human MMP-9 (diluted 1:50) and purified mouse monoclonal anti-human TIMP-1 (diluted 1:50) (Fuji Chemical Inc., Takaoka). Subsequently, the membrane was reacted with purified goat polyclonal peroxidase-conjugated anti-mouse immunoglobulin G antibody (diluted 1:1,000) as the 2nd antibody at room temperature for 1 h. The membrane was washed with PBS, and treated with enhanced chemiluminescence Western blotting solution. The enhanced chemiluminescence of protein bands was detected by exposing X-ray film to the membrane, and the expression of MMP-2, MMP-9 and TIMP-1 proteins was evaluated.

Statistical analysis

Using the StatView (Abacus Concepts, Inc., Berkeley, CA) software, the difference between 2 groups was tested by the Mann-Whitney *U* test. Differences among multiple groups were tested by the Kruskal-Wallis test, and the groups with significant differences were tested by the Tukey test (nonparametric test). The correlation between 2 groups was tested with Spearman's correlation coefficient by rank. A value of $P < 0.05$ was considered significant.

Results

Staging of AAA

The 23 patients with AAA (≥ 30 mm) consisted of 22 men and 1 woman. The mean age (± SD) was 72.4 ± 6.8 years, and the mean diameter (± SD) of aneurysms was 53.3 ± 10.8 mm. Of them, 6 patients (5 males and 1 female) had small AAAs (30–45 mm): their mean age was 70.3 ± 3.7 years and the mean diameter of aneurysms, 40.8 ± 5.6 mm (small-diameter AAA group). The remaining 17 patients (all male) had medium or large AAAs (> 45 mm): their mean age was 73.1 ± 7.8 years and the mean diameter of aneurysms, 57.5 ± 8.6 mm (medium-large-diameter AAA group).

The control group of 11 autopsy cases consisted of 7 males and 4 females aged 66.1 ± 14.1 years: the mean aortic diameter was 20.4 ± 2.4 mm. There were no significant differences among the 3 groups in clinicopathological profiles, except for aortic diameter, history of ischemic heart disease and malignant disease (Table 1).

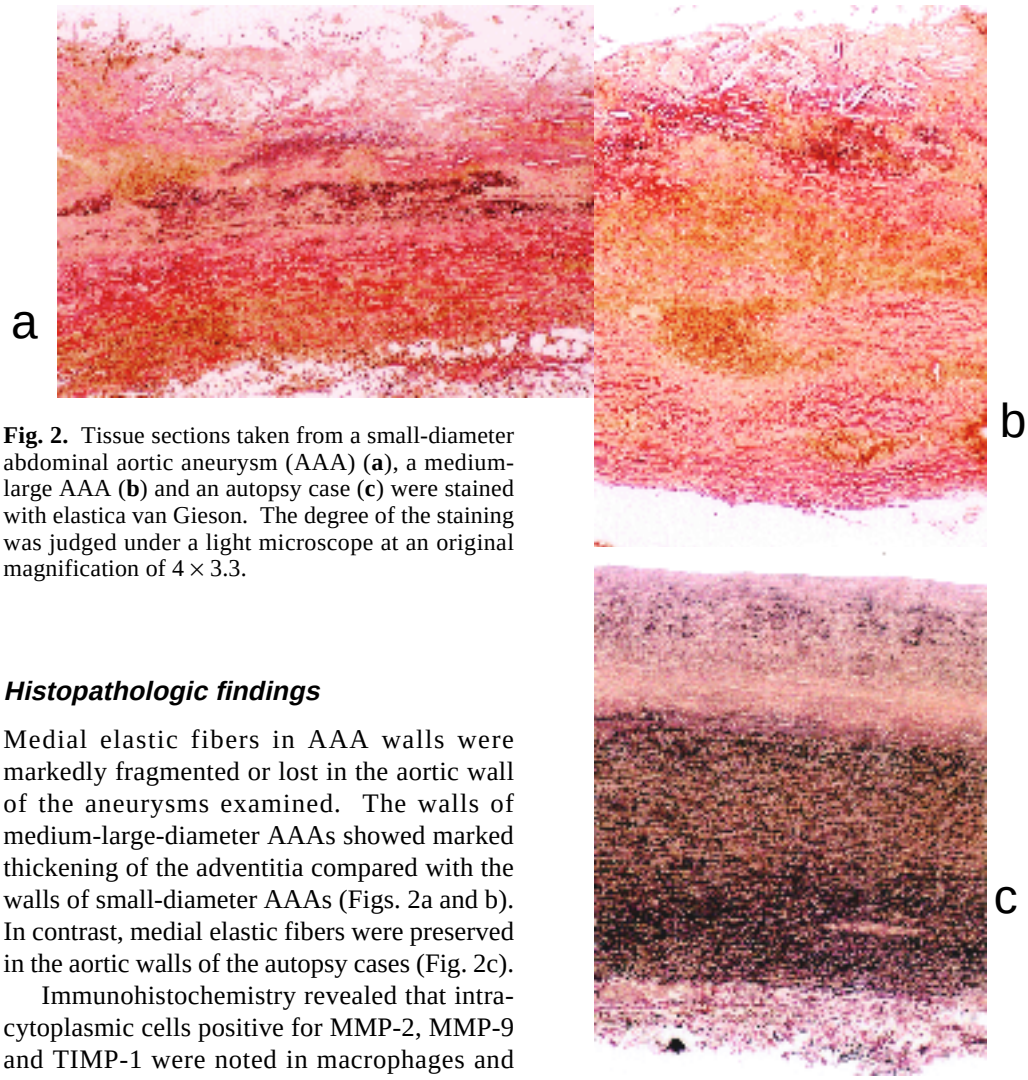

Fig. 2. Tissue sections taken from a small-diameter abdominal aortic aneurysm (AAA) (**a**), a medium-large AAA (**b**) and an autopsy case (**c**) were stained with elastica van Gieson. The degree of the staining was judged under a light microscope at an original magnification of 4×3.3.

Histopathologic findings

Medial elastic fibers in AAA walls were markedly fragmented or lost in the aortic wall of the aneurysms examined. The walls of medium-large-diameter AAAs showed marked thickening of the adventitia compared with the walls of small-diameter AAAs (Figs. 2a and b). In contrast, medial elastic fibers were preserved in the aortic walls of the autopsy cases (Fig. 2c).

Immunohistochemistry revealed that intracytoplasmic cells positive for MMP-2, MMP-9 and TIMP-1 were noted in macrophages and lymphocytes as well as vascular smooth muscle cells. MMP-2-positive cells were present in the intimal plaques and thickened intimas in the AAA walls (Fig. 3a). MMP-9-positive cells were present in the outer layer of the tunica media, adventitia and intimal plaques in the AAA walls (Fig. 3b). TIMP-1-positive cells were noted in the intimal plaques (Fig. 3c). HAM56-positive cells were seen in similar locations to MMP-9-positive cells in the AAA walls (Fig. 3d). A few cells positive for MMP-2, MMP-9 and TIMP-1 were also demonstrated in the control aortic walls.

Competitive PCR

Of the 34 specimens, messenger RNA (mRNA) could be obtained from 10 controls, 6 small AAAs and 14 medium-large AAAs. Figure 4 shows the expression ratios of MMP-2, MMP-9 and TIMP-1 to the internal control GAPDH. Table 3 summarizes the mean expression ratios of MMP-2, MMP-9 and TIMP-1 in the 3 groups. The MMP-2 expression ratio was the highest in the small AAAs, the value being significantly higher than that of the 10 controls ($P < 0.05$). The MMP-9 expression ratio was higher in the small AAAs and the medium-large

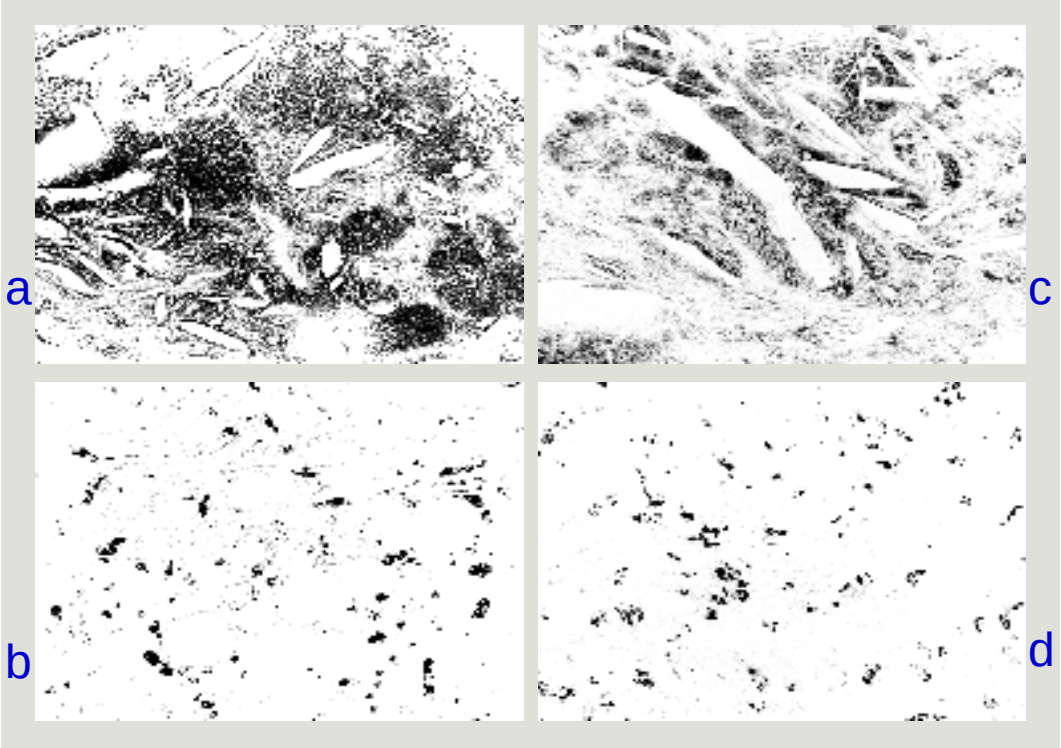

Fig. 3. AAA tissue shows a positive immunoreaction for matrix metalloproteinase (MMP)-2 (**a**), MMP-9 (**b**), tissue inhibitor of metalloproteinase (TIMP)-1 (**c**) and HAM56 (**d**). The degree of immunohistochemical staining was judged under a light microscope at an original magnification of 20 × 5.

AAAs than in the control group, *P* values being less than 0.01 and 0.05, respectively. On the other hand, there was no significant difference among the 3 groups on the TIMP-1 expression ratio, although the expression ratio tended to be higher in the small AAAs. Overall, the highest expression was noted in MMP-2, followed by TIMP-1 and MMP-9, in the order given.

The medium-large AAAs were divided into 2 subgroups by diameter; medium type < 65 mm and large type ≥ 65 mm. The mean expression ratio of MMP-9 was 0.026 ± 0.051 in the medium-type AAA [n = 11] and 0.006 ± 0.009 in the large-type AAA [n = 3], the value being higher in the former than in the latter without significant differences. The mean expression ratios of MMP-2 and TIMP-1 were 0.045 ± 0.073 and 0.334 ± 0.521 in the medium type and 0.281 ± 0.419 and 0.128 ± 0.190 in the large type, respectively. There was no statistical difference.

Table 4 shows the results of Spearman's coefficient by rank to examine the relationship of the expressions among MMP-2, MMP-9 and TIMP-1. The expression levels of MMP-9 and TIMP-1 signficantly correlated in all the AAAs and the medium-large AAAs ($P < 0.01$, Fig. 5), but not in the small AAAs and controls. Moreover, the expression levels of MMP-2 and MMP-9 also significantly correlated in a similar fashion ($P < 0.05$, Table 4). No significant correlation of expression was noted between MMP-2 and TIMP-1.

Western blotting

Protein could be obtained from a total of 9 specimens, 3 each from the 3 groups. MMP-2, MMP-9 and TIMP-1 were variably expressed as shown in Fig. 6. Of these, an obviously higher expression of MMP-2 and TIMP-1 was