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# Analysis of tissue inhibitor of metalloproteases (TIMP) as the unifying entity in the etiology of abdominal aortic aneurysms

Ellis Lorenzo Webster  
*Yale University*

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ANALYSIS OF TISSUE INHIBITOR OF METALLOPROTEASES (TIMP)  
AS THE UNIFYING ENTITY IN THE ETIOLOGY  
OF ABDOMINAL AORTIC ANEURYSMS

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YALE



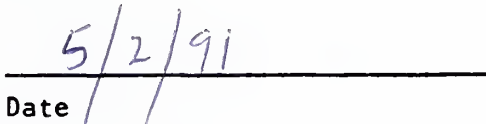
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ANALYSIS OF TISSUE INHIBITOR OF METALLOPROTEASES (TIMP) AS  
THE UNIFYING ENTITY IN THE ETIOLOGY OF ABDOMINAL AORTIC  
ANEURYSMS

Thesis Submitted to Yale University  
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of the Requirements for the Degree of  
Doctor of Medicine

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Ellis Lorenzo Webster

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# ANALYSIS OF TISSUE INHIBITOR OF METALLOPROTEASES AS THE UNIFYING ENTITY IN THE ETIOLOGY OF ABDOMINAL AORTIC ANEURYSMS.

Ellis L. Webster, Colleen M. Brophy,<sup>\*</sup> Thomas Barnett,<sup>+</sup> and M. David Tilson. Department of Surgery, Yale University School of Medicine, New Haven, CT & Department of Surgery, St. Luke's-Roosevelt Hospital, New York, NY. (Sponsored by Bauer Sumpio, Department of Surgery, Yale University School of Medicine, New Haven, CT).

<sup>\*</sup>Department of Surgery, New England Deaconess Hospital, Boston, MA.

<sup>+</sup>Molecular Diagnostics, Inc., West Haven, CT.

Aneurysmal disease is the 13th leading cause of death in the United States, and the incidence of the disease is increasing annually. The etiology of abdominal aortic aneurysms (AAA) is presently unknown, and several theories have been advanced in recent years. A 'minimum' requirement for a working hypothesis is that it should explain two important observations: 1) There is increased proteolysis of the aorta (which may be due to increased protease activity or decreased antiprotease activity); and 2) There is a strong familial predisposition, with 20-30% of first-degree relatives of patients with AAA also affected. A male predominance among AAA patients has also been noted. Tissue Inhibitor of MetalloProteases (TIMP) is a major antiprotease of connective tissue and its gene has been assigned to the X chromosome. Factors causing relative deficiency of this inhibitor could account for increased proteolysis and explain genetic features of the disease. Previous work suggests that TIMP is deficient in AAA aorta.



Experiments were performed to determine if deficiency of TIMP is due to mutation resulting in decreased expression or an altered gene product. Northern blots of RNA from dermal fibroblast cultures isolated from AAA patients were probed with human TIMP cDNA generated by polymerase chain reaction (PCR). Human TIMP cDNA generated from the isolated RNA in one patient was also sequenced. Dermal fibroblasts expressed TIMP mRNA in all cell lines and there was no significant difference in TIMP expression in six patients vs two controls. There was a normal nucleotide sequence of the cDNA.

These studies do not support the hypothesis that deficiency of TIMP in AAA tissue is due to a primary defect in the gene. Deficiency of TIMP may be due to decreased expression or binding under local conditions in the aneurysmal aorta or due to increased degradation. Further studies to rule out expression abnormalities in aortic smooth muscle cells and macrophages in AAA may be useful, since the present studies were based on dermal fibroblasts.



## Dedication

To all who believed in me, and made sacrifices on my behalf,  
especially my mother, Zelmera F. Webster, who insisted that I  
fulfill my dreams even when it seemed impossible.



## Acknowledgements

I would like to thank Dr. M. David Tilson for his passion for this subject and his mentorship while overseeing this project; Dr. Adrian Hayday and the members of his lab for the time, expertise, space, and supplies which they provided; Dr. Colleen Brophy, Dr. Tom Barnett, Dr. Olivier Kocher, Dr. Stephano Martinotti, and Dr. Peter Seperack for their contributions to the successful completion of this work.





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## Introduction

Aneurysms are described as local irreversible dilatation or outpouching of arterial walls with at least a 50% increase in diameter compared to the expected normal diameter [1,2]. They result in deterioration and destruction of the architecture of the arterial walls, resulting in loss of elastin and smooth muscle cells [3]. The abdominal aorta is the most common site of aneurysms in the aorta, and these are of two types, fusiform and saccular. Fusiform aneurysms are most common, and are cylindrical, involving the entire circumference of the aorta in the wall of the aneurysm. Saccular aneurysms, on the other hand, are rare and incorporate only a portion of the aortic wall into the wall of the aneurysm. As its name implies, this aneurysm is a sac-like protrusion from the aorta. [4]. Aneurysmal disease ranks 13th as a leading cause of death in the United States [5], and a recent epidemiological study reported that the apparent incidence of abdominal aortic aneurysms (AAA) has increased seven-fold between 1951 and 1980 [6]. Another study showed that 5% of the U.S. population over 60 years old have aneurysmal dilatation of the abdominal aorta, with an increase in incidence of new AAA of 8.7/100000 from 1950 - 60, to 36.5/100000 from 1971-80 [7].

Rupture of AAA is a life-threatening occurrence. Discovered before rupture, successful management can lead to long term survival with operative mortality less than 5%. However, if rupture occurs, the mortality increases to greater than 50%. [8,9]. Studies show that the 5 year risk of rupture is less than 15% in AAA up to 4



cm in diameter, and increases with increasing size of AAA to more than 75% at 8 cm. The rate of enlargement of AAA is approximately 0.4 cm per year. [7]. Identification of risk factors for AAA might permit early intervention for prevention and/or discovery of early asymptomatic lesions, amenable to elective repair. This would lead to decreased chance of rupture, and improve long term prognosis. Unfortunately, few factors predictive of the presence of AAA have been identified to date. In humans, AAA is predominantly a disease of elderly males [10], and population studies indicate that smoking, hypertension, and diet are important risk factors for development of these lesions [11]. However, screening a hypertensive population for AAA revealed aortic dilatation in only one of 245 patients [12]. It is known that certain strains of turkeys develop aortic aneurysms in males, associated with hypertension and abnormally increased levels of serum lipids, which are genetically determined in these species [13,14]. A better understanding of the etiology of AAA disease is, therefore, essential to more effective management of this disorder.

The etiology of AAA has met with some controversy. Until recently, atherosclerosis was considered to be the major etiologic agent for aneurysms, accounting for about 96%, with trauma, syphilitic aortitis, infectious agents (bacteria, fungi, mycobacteria), degenerative conditions (Marfan's syndrome, Ehlers-Danlos syndrome, and cystic medial necrosis), collagen-vascular diseases, various vasculitides, and congenital anomalies indicted in causing the remainder [2]. However, the atherosclerosis theory has been challenged because with experimental atherosclerosis, there has only been one aneurysm reported in dogs [15], and a single fusiform





aortic aneurysm among 730 squirrel monkeys [16], suggesting that atherosclerosis does not usually induce aneurysmal disease. Other observations which do not support the atherosclerosis theory are the rarity of aneurysmal development in aortas severely involved with atherosclerosis, and the infrequency of distal occlusive atherosclerotic lesions in patients with AAA [4]. Also, it has been shown that there are differences in the population characteristics, such as age, height, weight, sex, mortality and subsequent operative therapy, between patients with AAA and those with atherosclerotic occlusive disease, suggesting that there are differences in the underlying pathological processes [10]. This information has led many investigators to propose several hypotheses to explain aneurysmal development.

### Proteolysis hypothesis

The aorta is composed of elastin, arranged in the media in a concentric manner, a reticular network of type I and type III collagen fibers, and layers of smooth muscle cells. It was suggested that aneurysms may be secondary to a proteolytic process, since in aneurysmal aorta, elastin and collagen content is decreased, and there is increased collagenase and elastase activity, compared to normal aortas. [4]. Sumner *et al* [17] observed that the % collagen and % elastin in AAA is much less than in nonaneurysmal atherosclerotic aorta, and suggested that this is a reflection of the degenerative process involving the AAA wall. Collagen and elastin were more concentrated in the outer layer of the AAA. Many other investigators have defined proteolytic features of AAA disease, both



in serum and in tissue [18-21]. The deficit of collagen may be a precipitating cause of aneurysm formation and expansion. Dobrin *et al* [22] found that treatment of arteries with elastase resulted in dilatation, but remained intact; whereas treatment with collagenase resulted in rupture.

Busuttil *et al* [21] found that collagenase activity was detectable in the AAA, but not in atherosclerotic aorta, and collagenase activity correlated with aneurysm size. They hypothesized that endogenous collagenolytic activity may be responsible for aneurysmal expansion and rupture, and that the enzyme is localized in AAA wall and inoperative in atherosclerotic arteries. The degree of collagen breakdown correlates with AAA size. This collagenase-like activity may account for the decreased collagen found by Sumner *et al* in AAA tissue, and the relative absence of collagenase activity in atherosclerotic aorta could explain the greater content of collagen observed in this tissue. Measured collagenase activity is greater in the inner layer than in the outer layer, which would support Sumner's observation of more collagen in the outer layer.

Busuttil *et al* [23] also noted that elastase activity was increased in AAA, compared to atherosclerosis, while Cohen *et al* [19] demonstrated that aortic elastase was significantly higher in patients with AAA, compared to patients with occlusive aortic disease. These findings support the theory that aneurysm formation is secondary to a proteolytic process. Elastin content of aneurysmal aortic media is 8.1%; normal aortic media is 35%; and atherosclerotic aortic media is 22% [24]. The absence of collagenolytic and elastolytic activity in



atherosclerotic aorta not affected by aneurysmal change, points to the different pathogenesis between these 2 lesions [21].

The possibility of failure of a protease/antiprotease balance was suggested by Cannon and Read in 1982 [18]. Serum elastolytic activity and leucocyte elastolytic activity are significantly increased, with decreased antiproteolytic activity in AAA patients. One explanation of this would be that aneurysm of the aorta is associated with deficiency of antiprotease content. However, this condition has been reported only as a congenital defect by Eriksson [25], looking at protease and protease inhibition in chronic obstructive pulmonary disease (COPD). Another possibility is that even though the concentration and phenotype of  $\alpha_1$ -protease inhibitor is normal, its antiproteolytic capacity is not. This has been described by many investigators in cigarette smokers with and without emphysema [26-28]. Smoking is known to alter elastase/antiprotease balance in the lung, leading to emphysema [18,29]. Emphysema is thought to be secondary to proteolysis of the extracellular matrix of the lungs by macrophage elastases, which are inhibited by  $\alpha_1$ -antitrypsin in normal lung tissue [30-33].

Experiments by Cohen *et al* [19] support the protease/antiprotease imbalance theory, in that, comparing tissue from patients who underwent an operation on the abdominal aorta for ruptured AAA, an elective procedure for AAA, or aortofemoral bypass for occlusive disease, patients with ruptured AAA had the highest elastase activity, and the lowest  $\alpha_1$ -antitrypsin. This suggests that the homeostatic balance between elastase and



antiprotease may be significantly altered in the aortic wall, especially at the time of aortic rupture.

The proteolysis seen in AAA disease may not only be caused by an imbalance in elastase/alpha<sub>1</sub>-antitrypsin, but by other proteases not inhibited by alpha<sub>1</sub>-antitrypsin. Campa *et al* [24] report evidence of a metalloprotease which degrades elastin. They observed increased elastinolytic activity in AAA, predominantly secondary to a protease which hydrolyzed elastin, but is not inhibited by alpha<sub>1</sub>-antitrypsin or chloromethyl ketone, and has no immunological cross-reactivity with leucocyte elastase, which is a serine protease. This protease is inhibited by EDTA. Of note, there are only trivial amounts in atherosclerotic aorta, and none in normal aorta. Brown *et al* [20] and Reilly *et al* [34] have identified an elastolytic activity in AAA patients, with the inhibitory profile of a metalloelastase, which causes elastin degradation in normal aorta. This substance is not circulating elastase, and may originate from arterial aneurysmal tissue. It has affinity for aneurysm wall, is increased in patients with AAA, and is inhibited by copper. A similar metalloelastase has been isolated from macrophages in lung tissue [33], leading to the hypothesis that macrophages, present in the inflammatory infiltrate in AAA, release metalloelastases which are uninhibited by circulating antiproteases, resulting in the increased proteolytic activity observed in AAA disease [35]. Thus, the presence of increased proteolysis in AAA tissue is due to either an increase in the activity of proteases or a decrease in inhibitory activity [36].





### Biomechanical/physiological hypothesis

The abdominal aortic segment below the level of the renal outflow is particularly prone to the formation of both true aneurysms and atherosclerotic occlusive disease. Special local factors in the abdominal segment of the aorta in certain individuals may be of great importance in predisposing to the development of aneurysmal disease. [2]. Elastin content of aortic media decreases down the aorta from 50% in the thoracic aorta [24] to 24% in the infrarenal aorta [37]. Aneurysms develop in the region of the aorta with decreasing elastin content [24]. Also, smooth muscle cells in the infrarenal aorta have decreased replication potential in culture compared to thoracic aortic smooth muscle cells [3]. This limited ability to regenerate could account for the loss of architecture of the infrarenal aorta, resulting in aneurysm formation.

The aortic media is normally nourished by diffusion from the endothelial surface and by vasa vasorum, which penetrate into the media from the adventitial side. Diffusion from the lumen is sufficient to nourish the inner 0.5 mm of medial thickness. This zone contains no vasa vasorum and encompasses about 30 medial fibrocellular layers. Species with more than 30 medial layers have vasa vasorum beyond the 30th layer. The presence of medial vasa vasorum permits the medial layers to function at a higher level of medial stress, and, in large animals, the avascular inner zone maintains its orderly lamellar arrangement, while the outer vascularized zone may deviate somewhat from the usual architecture as the animal increases in size. Between the renal arteries and the aortic bifurcation, the adult human aorta is about 0.7 mm in



thickness. For mammals, in general, this thickness would be expected to include an outer vascularized zone of about 10 layers. The human abdominal aorta has 29 layers, and is devoid of medial vasa vasorum. [2].

The infrarenal abdominal aorta is a site at which medial tensile stresses are relatively elevated, and where the media is comparatively thin. Depending upon conditions, this location may be vulnerable to aneurysm formation. Transmission of flowing blood through a junction is dependent on the junctional cross-sectional area ratio (ratio of the sum of the cross-sectional areas of the branch vessels to that of the vessel of origin). There is minimal reflection of the pressure pulse with a cross-sectional area ratio in the range of 1.15 to 1.26 for a two-vessel branch. This is referred to as 'matched'. In most species, the infrarenal aorta to the common iliac system is matched with values greater than 1.15; however, humans are unmatched even at infancy with a value of 1.11. This value continues to decrease, averaging about 0.75 at age 50, contributing to continuous detrimental effects on the hemodynamics of the infrarenal aorta, and resulting in increased pulsatile reflections and increased lateral pressure. These effects are worsened in the presence of dilatation and aneurysm.

Measurements of elasticity and deformation indicate that the distal portion of the infrarenal aorta is the stiffest portion of the abdominal aorta, and is the primary site for receiving deleterious effects from dynamic disturbances of pulsatile reflection and lateral pressure. [2]



### Genetic/environmental hypothesis

There is evidence to suggest that aneurysms can be inherited in both 'mice and men'. Mice with certain alleles for coat color at the X chromosome locus, *Mottled*, have connective tissue abnormalities, which result in aortic aneurysms, and decreased tensile strength of skin [38-40]. These abnormalities in Blotchy mouse are a result of impaired formation of cross-links of collagen and elastin, secondary to failure to generate lysine-derived aldehydes required for cross-linking. The mutation affects copper metabolism [41,42], and since copper is a cofactor for lysyl oxidase [38] (the enzyme which converts lysine residues to aldehydes), it is likely that this copper deficiency results in inactivation of the enzyme, leading to increased collagen and elastin solubility. This pathogenesis coincides with that of aneurysms produced in turkeys [43,45] and rats [46] by administration of lathyrogens, such as  $\beta$ -aminopropionitrile (BAPN), which inactivate lysyl oxidase, resulting in decreased formation of lysine-derived aldehydes [2,4,43,44]. Copper deficiency has also been reported to produce aneurysms in poultry [47] and pigs [48].

In humans, there is familial tendency to AAA, suggesting a genetic etiology. A Swedish study reported that 18% of patients with AAA had at least one relative with AAA disease [49]. These findings were confirmed in a study of 250 patients with AAA, and 250 controls, where 19.2% of the patients with AAA had a first-degree relative with AAA, compared with 2.4% of the control subjects [50]. Two recent screening studies show that up to 29% of brothers of patients with AAA also have aortic dilatation or aneurysms [51,52]. Tilson & Seashore [53], in a study of 50 families with AAA in two or



more first-order relatives, observed that the number of men with AAA exceeded the number of women by ratios as high as 8:1. Bengtsson [51] also noted a male preponderance in AAA disease with 29% of brothers of patients with AAA also having aortic dilatation compared to 6% of sisters. These findings suggest an X-linked inheritance which would correspond to the Blotchy mouse model, where a known mutation on the X chromosome (affecting copper metabolism), predisposes to development of spontaneous aortic aneurysms. It is interesting that the AAA-specific elastase isolated by Brown [20] is inhibited by copper, and Tilson [54] documented decreased tissue copper levels in both experimental animals and patients with AAA.

Other evidence suggestive of a genetic predisposition to AAA includes the observations by Tilson & Dang [55] that patients with AAA tend to be taller, have a greater mean body surface area, and have generalized arteriomegaly. Also, AAA disease has been reported in identical twins [56]. Defects in collagen genes can account for some forms of aneurysmal disease [3]. A mutation in the gene for type III collagen in Ehlers-Danlos Type IV leads to thin skin and sudden rupture of large blood vessels. Also, a mutation in pro-alpha-2 gene for type I procollagen found in individuals with Marfan phenotype leads to sudden rupture of the aorta [57]. Kontusaari *et al* [58] report the development of aortic aneurysms in a family caused by a single base mutation in type III procollagen gene. A single base mutation of glycine 619 of alpha<sub>1</sub> (III) chain to a codon for arginine resulted in synthesis of procollagen which undergoes





thermal unfolding at a lower temperature, leading to mild bleeding tendencies and aortic aneurysm formation.

Powell *et al* [59] have suggested that a gene on chromosome 16 is associated with AAA. Investigation of polymorphic serum markers show an increased frequency of the haptoglobin alpha<sub>1</sub> gene in patients with aneurysms [60]. The haptoglobin gene is located on the long arm of chromosome 16, close to 2 genes coding for proteins involved in lipid metabolism; *lethicin : cholesterol acyl transferase* (LCAT) and *cholesterol ester transfer protein* (CETP). CETP is polymorphic, and the frequency of rare polymorphisms at the CETP locus is increased in AAA patients. Haptoglobin functions by binding hemoglobin and transporting it to the liver during hemolysis. Haptoglobins containing the alpha<sub>1</sub> chain accelerated the degradation of aortic elastin by elastases *in vitro* by two- to four-fold, probably by binding to elastin. They concluded that genetic variation in haptoglobin and CETP genes appears to influence dilatation of the abdominal aorta. Also, variation at the haptoglobin locus has a direct effect on elastin degradation, and CETP variation could affect lipid metabolism and promote atherosclerosis. Thus, they suggested that a gene on chromosome 16 is associated with AAA, although this may be an as yet unidentified gene in linkage equilibrium with the haptoglobin and CETP genes. An autosomal pattern of inheritance for some AAA disease would be consistent with earlier reports by Tilson & Seashore [53] that 12 of 50 families with AAA had father to son transmission, suggesting an autosomal dominant pattern of inheritance.



### TIMP hypothesis

Proteolysis, coupled with the biomechanical and physiological properties of the abdominal aorta, can account for AAA development, but do not explain the selective increase in AAA disease seen in relatives of patients with AAAs. The relative risk to brothers and sisters of patients with AAA is significantly greater than 1 indicating that familial factors are important in the etiology of AAA [61]. It appears that the etiology of AAA is related to a mechanism that links the proteolytic and genetic phenomena observed in AAA disease. Assignment of a gene coding for a Tissue Inhibitor of Metalloproteases (TIMP) to the X chromosome raises the possibility that a relative deficiency of this factor might be the link between proteolytic and genetic phenomena [62,63]. TIMP is a molecule of 28.5 kDa that has been implicated as a tissue inhibitor of collagenase, gelatinase, proteoglycanase, and other metalloproteases [33,64,65]. It was localized to the X chromosome from Xp 11.1 to Xp 11.4 by *in situ* hybridization [62]. As stated earlier, there is evidence to suggest X-linked inheritance of AAA disease, with the number of men with AAA exceeding the number of women by ratios as high as 8:1 [53]. The presence of an inhibitor of metalloelastase on the X chromosome suggests that predisposition to a relative deficiency of TIMP could result in a protease/antiprotease imbalance, leading to proteolysis and AAA, predominantly in males. If this predisposition is detectable by restriction fragment length polymorphisms (RFLPs) in patients with AAA, it would be possible to screen high risk populations, leading to early intervention in AAA disease. Durphy *et al* [66] reported that BglII identifies a two allele polymorphism in the



human TIMP gene. The frequency of the 12 kb allele is .66, and the 9.5/2.5 kb allele is .34. Recent studies by Reilly & Tilson [personal communication] show that the size and frequency of these alleles are the same in patients with AAAs.

TIMP can be relatively deficient in tissue if it is defective, down-regulated, or inactivated. There are reports of low levels of TIMP and increased protease activity in bone cultures induced to undergo resorption [67], and in rabbit synovium cultures taken from arthritic rabbits [68]. Also, there is evidence that TIMP serves to regulate collagenase activity during collagen turnover in many connective tissues throughout the body [69]. Brophy *et al* [70] show that there is decreased TIMP in the aortas of patients with AAAs, measured by Western blot and radioimmunoassay. These findings support the TIMP hypothesis as the unifying theory between proteolysis and sex-limitation in AAA disease.

The purpose of this investigation is to: 1) look at expression of TIMP in different cell types, 2) sequence the TIMP gene from patients with AAA, and 3) evaluate expression of the TIMP gene in patients with AAA, compared to controls. It would be useful to identify a genetic marker such as a mutation in the TIMP gene or a regulatory defect which can be used to develop simple, cost-effective screening tests so that individuals at high risk for AAA disease can be easily identified, even prior to aneurysm development. It is encouraging to note that the mutation reported by Kontusaari, which predisposes to some aortic aneurysms, can be detected in DNA from saliva [58].



- 1) Edwards *et al* [71] reported that exposure of quiescent human fetal lung fibroblasts to growth factors in the presence of Transformation Growth Factor- $\beta$  (TGF- $\beta$ ) resulted in inhibition of collagenase induction, and a synergistic increase in TIMP expression. Experiments were performed to examine the effect of TGF- $\beta$  on TIMP expression in fibroblasts, smooth muscle cells, and aortic endothelial cells, in an effort to extrapolate Edwards' findings to cells more likely to be involved in aneurysm development.
- 2) A relative deficiency of TIMP could be secondary to a mutation in the TIMP gene, resulting in a defective product, which is unable to inhibit elastin degradation. The TIMP gene from patients with AAA was sequenced and compared with the normal sequence [72].
3. Down-regulation of the TIMP gene could result in decreased transcription, resulting in decreased levels of TIMP and increased proteolysis. Reports that TIMP is decreased in patients with AAA support this hypothesis [70]. Experiments were performed to compare TIMP expression in patients with AAA and patients without AAA.





## Materials and Methods

### TIMP expression in various cell types

Total RNA from rat fibroblast and smooth muscle cells, and bovine aortic endothelial cells was obtained from Dr. Olivier Kocher in Dr. J. Madri's laboratory (Department of Pathology, Yale University). Cells were treated with transforming growth factor- $\beta$  (TGF- $\beta$ ). Untreated cells were used as controls. 10 micrograms ( $\mu\text{g}$ ) of total RNA was electrophoresed in a 1.2% agarose gel containing formaldehyde as described in Sambrook *et al* [73]. The gel was made by melting 1.8 g agarose in 15.2 ml 5X Running Buffer (200 mM MOPS, 50  $\mu\text{M}$  sodium acetate, pH 7.0, 10  $\mu\text{M}$  EDTA, pH 8.0) and 60.8 ml ddH<sub>2</sub>O, cooling to 56°C, and adding 9.1 ml formaldehyde, 15.2 ml 5X Running Buffer, and 52.7 ml ddH<sub>2</sub>O. 7.5  $\mu\text{l}$  Ethidium bromide (10mg/ml) was added to a final concentration of 0.5  $\mu\text{g}/\text{ml}$ . 10  $\mu\text{l}$  loading buffer (stock solution = 250  $\mu\text{l}$  formamide, 100  $\mu\text{l}$  RNA-Bond Dye, 80  $\mu\text{l}$  formaldehyde) was added to 10  $\mu\text{g}$  total RNA and loaded with micropipetting onto the gel immersed in 2 L of 1X Running Buffer. The gel was electrophoresed at 150 volts for 10 min, and then at 40 volts for 12 hours. The gel was then soaked in 10X SSC for 10 min, marked for orientation, photographed, and allowed to soak for 20 min more to wash out excess ethidium bromide. The gel was inverted onto a sheet of oversized Whatmann's paper, and a nitrocellulose filter of exact size was placed on top of the gel, with care taken to avoid air bubbles. This was covered by 4 sheets of Whatmann's paper and a 2 inch stack of paper towels cut to exact size. A glass plate and weight was placed on top, and RNA transfer to



the nitrocellulose filter was allowed for 5 hours, using 20X SSC as the transfer solution. The filter was air-dried and baked at 80°C, under vacuum, for 2 hours.

### Preparation of TIMP Probe for Hybridization

The probe used for hybridization was mouse TIMP cDNA (16c8) kindly provided by Dr. D. Denhardt (University of Western Ontario). A maxiprep of the plasmid containing the 16c8 TIMP cDNA was performed. Cells containing the plasmid DNA were spread onto LB-agar plates containing Ampicillin and incubated at 37°C overnight. Two of the colonies were added to 2 ml LB+Amp broth and placed at 37°C for 12 hours, with shaking (325 RPM). These 2 ml cultures were added to 500 ml of LB+Amp broth in a 2 L flask, and incubated at 37°C overnight, with shaking.

400 ml of the culture was placed on ice for 15 min and then centrifuged at 5000 RPM for 15 min. The supernatant was discarded and the pellet was resuspended with vigorous pipetting in 16 ml of Solution I (3.6 ml 50% glucose solution, 2.5 ml 2 M Tris-HCl, pH 8.0, 4 ml 0.5 M EDTA, pH 8.0, and 189 ml ddH<sub>2</sub>O) and 50 mg of Lysozyme. This suspension was left at room temperature for 10 min and then mixed with 32 ml of Solution II (10 ml 20% SDS, 8 ml 5 M sodium chloride, and 182 ml ddH<sub>2</sub>O), and placed in an ice-H<sub>2</sub>O bath for 5 min. 16 ml of pre-cooled Solution III (60 ml potassium acetate, 11.5 ml glacial acetic acid, and 128.5 ml ddH<sub>2</sub>O) was added and placed in ice-H<sub>2</sub>O bath for 5 min. The mixture was spun at 5000 RPM for 15 min. The supernatant was filtered through gauze and spun again at 5000 RPM for 15 min. 0.6 volumes of isopropanol was added and left at



-20°C for 15 min. The solution was spun at 6000 RPM for 15 min at 0°C, drained well, and the pellet was resuspended in 10 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) with Tris-HCl, pH 8.0 added until the pH of the solution was greater than 6.5.

10 g of Cesium chloride was added to the suspension and it was transferred to a 30 ml ultracentrifuge tube, which was topped-up with a CsCl:TE solution (1:1 w/v) and 0.5 ml ethidium bromide (10 mg/ml). This mixture was spun at 48000 RPM for 12 hours at 15°C. The DNA band was extracted with a 18-gauge needle and transferred to a 5 ml ultracentrifuge tube. Ethidium bromide was added and this solution was spun at 55000 RPM for 12 hours at 15°C.

The DNA band was extracted as before and transferred to a 15 ml Falcon tube. Ethidium bromide was removed by extracting with one volume of 90% butanol, vortexing, and discarding the top phase. This was repeated six times until the DNA solution was clear. The solution was placed in a dialysis bag and submerged in 2 L TE with stirring for 30 min. This was repeated twice and the OD<sub>260/280</sub> was checked.

### Labeling of TIMP cDNA Probe for Hybridization

5 µg of plasmid DNA containing the mouse TIMP cDNA (16c8) was linearized by digestion with PvuII at 37°C for 1 hour. PvuII cuts in the polylinker of the plasmid DNA. 10 µl of the digest was electrophoresed on a 1.5% agarose minigel to evaluate efficiency of digestion. DNA was radiolabeled by modification of the method of oligolabeling described by Feinberg & Vogelstein [74]. 60 ng of linearized plasmid DNA containing the mouse TIMP cDNA was added



to 32.5  $\mu\text{l}$  ddH<sub>2</sub>O and placed at 95°C for 5 min, 37°C for 5 min, and then on ice. This solution was incubated with 10  $\mu\text{l}$  Oligolabeling Buffer (-dCTP) [74], 2  $\mu\text{l}$  bovine serum albumin (10 mg/ml), 5  $\mu\text{l}$  <sup>32</sup>P-dCTP, and 2 U Klenow DNA polymerase I, at room temperature overnight. The reaction was stopped with 50  $\mu\text{l}$  STE buffer and purified through a Sephadex G-50 spun column [73]. The column was prepared by plugging the tip of a 1 cc syringe with glass wool, filling with Sephadex G-50 solution, and spinning in a 15 ml Falcon tube until the G-50 column was compact. The column was washed twice with 200  $\mu\text{l}$  of STE, with spinning. The labeling reaction mixture was extracted with phenol:chloroform, and the aqueous phase was run through the column. The sample was recovered (~100  $\mu\text{l}$ ), checked with a Geiger counter, and used for hybridization.

### Prehybridization and Hybridization

The baked filter was wetted in 2X SSC and placed into a hybridization slot containing 15 ml of prehybridization solution (3.75 ml 20X SSC, 7.5 ml formamide, 3.0 ml 50X Denhardt's Solution, 0.5 ml sodium phosphate, 0.5 ml 20% SDS, and 250  $\mu\text{l}$  salmon sperm DNA boiled for 10 min) at 42°C for 2 hours. The prehybridization solution was poured off, and 15 ml of hybridization solution (3.75 ml 20X SSC, 7.5 ml formamide, 0.5 ml 50X Denhardt's Solution, 0.2 ml sodium phosphate, 0.5 ml 20% SDS, 3.0 ml Dextran sulfate, and 250  $\mu\text{l}$  salmon sperm DNA and 100  $\mu\text{l}$  labeled TIMP cDNA boiled for 10 min) was added, and left at 42°C for 20 hours. The filter was transferred into 2X SSC to remove extremely loose counts, and washed as follows: twice in 1 L 2X SSC/0.1% SDS at 42°C for 20 min each, and three





times in 0.6X SSC/0.1% SDS at 42°C for 30 min each. The filter was placed onto Whatmann's paper, air-dried, covered with Saran wrap, and exposed to Kodak autoradiography film at -70°C for 48 hours. The film was developed and the blots of TIMP hybridization were scanned to determine the density of TIMP expression using a density scanner in Dr. S. Altman's laboratory (Department of Biology, Yale University).

### RNA isolation from human fibroblasts

Fibroblast cultures were established from abdominal skin obtained during the incision for aortic reconstruction in six patients with aneurysm disease, and one control patient with occlusive disease. The skin specimens were washed in phosphate buffered saline (PBS), 70% isopropanol, and again in PBS, then defatted, minced, placed dermal side down on petri-dishes, and allowed to dessicate for 45 minutes. Dulbecco's modified eagles medium supplanted with 10% fetal bovine serum (Gibco, Grand Island, NY) and 10,000 U/ml penicillin/streptomycin (Gibco) was then added. The cells were allowed to reach confluence (approximately one month), passaged with 0.25% trypsin, and plated onto 150 cm<sup>3</sup> dishes. When confluent, the cells were washed with 10 ml PBS X 2 and 3.5 ml 4 M GIT solution (guanidium isothiocyanate (Boehringer), 20 mM sodium acetate pH 5.2, 0.5% N-lauryl sarcosine,  $\beta$ -mercaptoethanol) was added. The viscous solution was then drawn up via a 21-guage needle into a 10 cc syringe and drawn up and down 4 times. The GIT solution/cell lysate was layered onto 1.5 ml of 5.7 M CsCl in ultracentrifuge tubes, and centrifuged in a Beckman



L8-70M at 35000 RPM for 18 hours at 18°C. The supernatant was aspirated and the pellet was resuspended in 200 µl TES (10 mM Tris pH7.4, 5 mM EDTA, 1% SDS). A chloroform/butanol (4:1) extraction was performed X 2. 40 µl 3 M sodium acetate and 1 ml 95% EtOH were added to the final 360 µl RNA/TES. This was placed at -80°C for 2 hours, and then centrifuged at 14000 RPM for 30 minutes at 4°C. 360 µl of water was added and 10 µl of this solution, plus 490 µl water was added to determine OD<sub>260/280</sub>. 40 µl of 3 M sodium acetate and 1 ml 95% EtOH were added, and the RNA was stored at -80°C as a suspension.

#### Sequencing of TIMP gene in AAA

Total fibroblast RNA isolated from AAA patient #2 (figure 3,4) was prepared as above. Complementary DNA (cDNA) was transcribed from 2 µg of total RNA in a 50 µl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl<sub>2</sub>, 500 mM each of all four deoxynucleotides, 2.5 µg random hexamers (pdN<sub>6</sub>), 1 U placental ribonuclease inhibitor and 800 U recombinant Moloney murine leukemia virus reverse transcriptase (Bethesda Research Labs, Gaithersburg, MD). Incubation was at 37°C for 1 hour.

#### Polymerase Chain Reactions (PCR)

Oligonucleotide primers were synthesized by Dr. T. Barnett (Molecular Diagnostics, Inc., West Haven, CT). PCR for TIMP cDNA-specific amplification was performed by incubating 5 µl of cDNA in a 100 µl reaction mix with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 200 µM each dNTP, 1 µM each TIMP



oligonucleotide primer, and 25 U/ml AmpliTaq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT). Primer pairs used for amplification were 5' forward oligo TIMP1 (5'AGATCTAGCGCCAGAGACACCAGAGA3'), 3' reverse oligo TIMP2 (5'GGGATCCGGGTGGACACTGTGCAGGCTTC3'), and internal forward oligo TIMP3 (5'GGGATCCAAGCCTTAGGGGATGCCGCTGA3'); restriction endonuclease cleavage sites for BglII (AGATCT) or BamHI (GGATCC) are incorporated at the 5' ends of the appropriate oligonucleotide. Amplification in a Perkin/Elmer thermocycler was for a total of 40 cycles as follows: cycle 1 (94°C, 5 min; 55°C, 2 min; and 72°C, 3 min), cycles 2 through 39 (94°C, 1 min; 55°C, 2 min; and 72°C, 3 min) and cycle 40 (94°C, 1 min; 55°C, 2 min; and 72°C, 8 min). Products of PCR reaction were analyzed by electrophoresis of 5 µl on 1.5% agarose/1.5% NuSieve agarose gels.

### Cloning and Sequencing of PCR Products

PCR reactions prepared by priming of cDNA with oligos TIMP1 and TIMP3, or with TIMP2 and TIMP3, were extracted by phenol/chloroform, ethanol precipitated and resuspended for digestion with restriction endonucleases BamHI and BglII (TIMP1/3 product) or BglII alone (TIMP2/3 product), then electrophoresed on 1% LGT (low gelling temperature)-agarose. Ethidium bromide staining bands corresponding to TIMP1/TIMP3 products (~700 bp) and TIMP2/TIMP3 products (~450 bp) were excised, melted with 0.3 M sodium acetate and 0.1 M Tris-HCl, mixed with water-saturated phenol and cooled on ice. Separation of agarose and phenol layers from aqueous was by centrifugation. DNA in the upper layer was



precipitated by addition of two volumes of ethanol and frozen in dry-ice. Pelleted DNA was resuspended in 5  $\mu$ l H<sub>2</sub>O. DNA segments were then prepared for sequencing by ligation with BamHI-cleaved Bluescript KS+ plasmid vector DNA (Stratagene, San Diego, CA). Reactions were 100 ng of cleaved DNA, 2 ng BamHI-cleaved vector DNA, 10 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM MgCl<sub>2</sub>, 2 mM ATP, and 400 U T4 DNA ligase (New England Biolabs, Beverly, MA) in 15  $\mu$ l at 15°C overnight. Transformation of ligated DNA was into competent *E. coli* DH5-alpha cells spread onto LB-agar plates containing 75  $\mu$ l of 2% X-gal and 15  $\mu$ l of 100 mM IPTG (isopropylthiogalactoside). Potential recombinant candidates were picked from among the white colonies on the plates and grown in 2 ml LB broth at 37°C overnight.

Minipreps of plasmid DNA were performed. 1.4 ml of 2 ml cultures was pipetted into eppendorf tubes and spun for 2 min. The supernatant was discarded and the pellet resuspended in 300  $\mu$ l STET buffer (8% sucrose, 5% Triton X100, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA). 25  $\mu$ l Lysozyme (10 mg/ml) was added to each tube which were vortexed for 2 min, boiled for 1 min, and spun for 15 min. 200  $\mu$ l of supernatant was mixed with 230  $\mu$ l isopropanol, placed at -20°C for 30 min, and spun for 5 min. The pellets were resuspended in 50  $\mu$ l TE buffer (10 mM Tris-HCl, 8.0, 1 mM EDTA), extracted with phenol X2, CHCl<sub>3</sub> X1, and precipitated with 0.3 M sodium acetate, pH 7.0, and two volumes of ethanol in a dry-ice bath. Tubes were spun for 10 min and the DNA pellets were washed with 70% ethanol in a speed-vac. 50  $\mu$ l TE and 1  $\mu$ l RNase (10 mg/ml) were added to each tube, followed by incubation at 70°C, 10 min, and





37°C, 15 min. Recombinant clones were determined by digestion of plasmid DNA at flanking sites with EcoRI/XbaI, since BglII termini cannot be excised after ligation to complementary BamHI ends. Plasmid DNAs demonstrating inserts of the expected sizes (either ~700 or 400 bp) were sequenced in both directions directly from double-stranded DNA by the dideoxynucleotide method of Sanger *et al.*, described in [73]. The primers employed for sequencing directly from Bluescript DNA were T7 and T3 in the presence of <sup>35</sup>S-dATP (Amersham Corp.). Sequence analysis was performed using the University of Wisconsin Genetics Computer Group programs.

#### TIMP expression in AAA

Total fibroblast RNA from six AAA patients and one patient with occlusive disease was prepared as described above. 15 µg aliquots of total RNA from each cell line was electrophoresed in a 1.2% agarose gel containing formaldehyde as described above. 15 µg of total RNA from a lymphoblast cell line which does not express the TIMP gene was obtained from Dr. Stefano Martinotti in Dr. A. Hayday's laboratory (Department of Biology, Yale University) and used as a negative control (figure 3A,3B, #8). A second gel was run with 10 µg aliquots of total RNA from AAA patients (#1, 2, 6, and 7), the patient with occlusive disease (#5), and total RNA from a normal human fibroblast cell line donated by Dr. P. Seperack (Miles Pharmaceuticals, West Haven) which was used as a second positive control (figure 4A,4B, #9). RNA was transferred to nitrocellulose filters, and prehybridized and hybridized at 42°C in prehybridization and hybridization solutions as previously described. Blots were



washed to a stringency of 0.1X SSC/0.1% SDS at 42°C, air-dried, and autoradiographed at -70°C for 36 hours.

The probe used for hybridization was human TIMP cDNA, generated from total fibroblast RNA from AAA patient #2, and amplified via PCR, as described earlier. Bluescript plasmid containing the PCR product from oligos TIMP 2/TIMP3 was linearized by digestion with BamHI at 37°C for 1.5 hours. 60 ng of linearized DNA was radiolabeled and purified as previously described.



## Results

Total RNA from rat fibroblasts and smooth muscle cells, and bovine aortic endothelial cells was probed with mouse TIMP cDNA to detect TIMP expression in different cell types. The TIMP gene is expressed in fibroblasts, smooth muscle cells, and aortic endothelial cells (figure 1A). TIMP expression is increased two- to three-fold in cells exposed to TGF- $\beta$  (figure 1B).

Human TIMP cDNA was generated from total RNA isolated from dermal fibroblast cell lines of patients with AAA. TIMP cDNA was amplified via PCR, cloned into Bluescript KS+ plasmid vector DNA, and sequenced in both directions directly from dsDNA by the chain-termination dideoxynucleotide method of Sanger *et al.* The sequence of the TIMP cDNA derived from cell line #2 (figure 3,4) was identical in nucleotide, and expected amino acid sequence (figure 2B) to that published by Docherty *et al* [72].

Dermal fibroblasts cultures were established from patients with AAA and a positive family history for AAA. Five of the six patients were male, and ages ranged from 67 years to 77 years. The only surviving control cell line (figure 3,4, #5) was established from an age-matched female patient who underwent abdominal surgery for occlusive disease. Total RNA from a normal human fibroblast cell line was donated by Dr. P. Seperack (Miles Pharmaceuticals, West Haven) and used as a second positive control (figure 4A,4B, #9). RNA was probed with human TIMP cDNA to compare TIMP expression in AAA vs. controls. Densitometry studies were performed in Dr. S. Altman's laboratory (Department of Biology, Yale



University). TIMP is expressed in the fibroblast cell lines, but not in equivalent amounts of the lymphoblast cell line (figure 3A,3B). There is no significant difference in TIMP expression in patients with AAA compared to patients without AAA (figure 3,4).





## Discussion

The results in figure 1 are consistent with the findings of Edwards *et al* that TIMP is expressed in fibroblasts, and there is increased expression in the presence of TGF- $\beta$ . The result most relevant to this paper is that TIMP is present in smooth muscle cells and aortic endothelial cells and can be regulated by growth factors. In order to implicate TIMP in AAA development, it must be present in the cells most directly affected by AAA formation, and have the ability to be regulated. These results show that both these criteria are met.

Figure 1A shows an abundance of nonspecific binding of the probe resulting in the bands in the region of TIMP mRNA being less discernable. Since the nonspecific binding is confined to the lanes of transferred RNA, it is most likely secondary to complementary sequences in the plasmid DNA used to clone the mouse TIMP cDNA 16c8. The plasmid was only linearized prior to labeling. The decreased intensity of the signals in the region of TIMP expression could also be due to the level of stringency used while washing the filter. The TIMP gene has approximately 70% conserved sequences across species, therefore, a higher level of hybridization would be expected than was observed. It is possible that the high concentration of the cloning plasmid DNA resulted in decreased TIMP specific labeling. The TIMP cDNA was ~600 bp, whereas the plasmid DNA was almost 2000 bp. It would have been better to cut the TIMP sequences out of the plasmid to allow a higher concentration of



labeled TIMP DNA, resulting in more specific hybridization, and a cleaner northern blot.

Figure 2B shows that the sequence of TIMP cDNA from one AAA patient is identical to the normal sequence previously published. It would be useful to look at the sequence of some of the other TIMP cDNAs to confirm that there is no sequence change in any of the cultured AAA cell lines. Using PCR to amplify the TIMP cDNA has potential for error since early misincorporation of one or more bases could be retained and propagated through succeeding cycles of amplification, resulting in an increased frequency of error products. The use of Taq DNA Polymerase reduces the possibility of errors since Taq DNA Polymerase has high fidelity, with a misincorporation rate/cycle of  $2 \times 10^{-4}$ , and only highly complementary substrate remains as the reaction approaches the temperature at which catalysis occurs [75]. PCR based on Taq DNA Polymerase represents a form of "cell-free molecular cloning" that can accomplish amplification in 3-4 hours which might take days or weeks of biological growth and biochemical purification [75].

Figures 3 and 4 show no significant difference in TIMP expression in dermal fibroblasts from AAA patients compared to patients without AAA. Evidence of decreased TIMP expression in AAA would support the hypothesis that decreased TIMP expression accounts for the observed TIMP protein deficiency in aneurysmal aorta tissue. However, the higher level of TIMP expression seen in the control (figure 3B, #5) is not significant since the amount of variation is within the accepted range. Also, when the filter was probed with a human tubulin gene PCR product (395 bp) to evaluate



the efficiency of the hybridization technique, the intensities of the blots showed comparable variation as seen when probed with TIMP. Thus, the difference observed by densitometry studies in figure 3B is not significantly real, and may be a result of better loading of the gel or better transfer of RNA to the nitrocellulose filter in the case of #5. Evidence of similar TIMP expression in AAA and nonAAA patients is confirmed in figure 4B. Unfortunately, these results are based on only two controls, and it is not possible to state undoubtedly that AAA patients do not have down-regulation of TIMP expression. It would be helpful to have more normal and nonAAA controls to confirm these results.

The deficiency of TIMP gene product in AAA which has been observed [70], may be secondary to loss of elastin, since TIMP is a matrix-associated protein [76], and there is decreased matrix protein in AAA [17,24,37]. Another possibility is that TIMP deficiency is site-specific, and localized to the infrarenal aorta. Further studies with aortic smooth muscle cells and *in situ* hybridization using abdominal aortic tissue would help to evaluate these possibilities. Dermal fibroblasts were used in this study because most of the work on TIMP expression has been done using dermal fibroblasts, and it would have been useful to detect a systemic defect in AAA disease, which would facilitate the development of a simple, cost-effective screening technique. Also, abdominal aortic smooth muscle cells have decreased replication potential [3] and it is difficult to determine whether findings are primary, or merely secondary features of AAA.



## **Conclusion**

Aneurysmal disease is the 13th leading cause of death in the United States and the incidence of the disease is increasing annually [5-7]. Rupture of AAA is a life-threatening occurrence with mortality up to 80% [61]. Several hypotheses have been proposed to explain AAA development including proteolysis, biomechanical/physiologic phenomena, and genetic predisposition. There is a considerable amount of convincing evidence in support of proteolytic and genetic phenomena. It would be useful to discover a unifying entity between these two hypotheses. Assignment of TIMP to the X chromosome raises the possibility that a relative deficiency of this inhibitor of metalloproteases may be the link between the increased protease activity and the male preponderance observed in AAA disease. Studies describing decreased antiprotease inhibitory activity [18,19] and decreased TIMP in AAA tissue [70] support this hypothesis. It was felt that the apparent low incidence of AAA in women may be due to a gene dosage effect, where the TIMP gene escapes inactivation on either X chromosome [36]. Genes have been studied at the region on the short arm of the X chromosome where TIMP has been localized which escape inactivation, and are expressed from both X chromosomes [77]. Recent work has shown that there is a 100-fold less transcription of the TIMP gene from the inactive X chromosome than from the active X suggesting that the TIMP gene is inactivated on one X chromosome in females, thus ruling out the gene dosage hypothesis [78].





TIMP is expressed in various cell types (figure 1) including fibroblasts, smooth muscle and aortic endothelial cells. TIMP is also present in skin and corneal fibroblasts, uterine smooth muscle cells, ovaries, fetal osteoblasts, tendon and cartilage explants, aortic smooth muscle cells, serum and amniotic fluid [69]. This ubiquitous nature of TIMP has implicated it as a major factor in the regulation of connective tissue turnover throughout the body [69].

The presence of a primary defect in the TIMP gene could result in a defective gene product with decreased inhibitory function, or in down-regulation of the gene leading to decreased expression and a deficiency of the inhibitor in tissue. Detection of such a defect could be useful to a better understanding of the disease process and development of a screening test to identify high risk populations, thus allowing earlier intervention for prevention or elective repair of AAA. The present studies do not support the hypothesis that the deficiency of TIMP in aneurysmal aorta is due to a primary defect in the gene, since there is no apparent sequence change, and no significant difference in TIMP transcription in dermal fibroblasts. The deficiency of TIMP gene product in AAA [70] may be secondary to loss of elastin, since TIMP is a matrix-associated protein [76], and there is decreased matrix protein in AAA [17,24,37]. Another possibility is that TIMP deficiency is site-specific, and localized to the infrarenal aorta. Abdominal aortic smooth muscle cells have decreased replication potential [3] and the effect of factors which inhibit or degrade TIMP would be potentiated since there is decreased turnover of cells. Further studies with aortic smooth muscle cells and *in situ* hybridization using abdominal aortic tissue



may be helpful, although it will be difficult to determine whether findings are primary, or merely secondary features of AAA.

The decrease in TIMP in AAA tissue may be due to an increase in neutrophil elastase activity [70]. TIMP can be degraded by neutrophil elastase and other serine proteases [80], and Cannon [18] and Cohen [79] have shown that neutrophil elastase activity is increased and antiproteolytic activity decreased in AAA. Thus, neutrophil elastase may potentiate metalloprotease activity by inactivation of its inhibitor. It is possible that aneurysm patients produce normal concentrations of TIMP that is less biologically active, i.e., TIMP that is less able to associate with the matrix, less able to interact with its binding-site, or more susceptible to degradation. This phenomena has been reported in cigarette smokers with and without emphysema, where a normal concentration and phenotype of  $\alpha_1$ -antiprotease is present, but antiproteolytic capacity is not [26-28]. Thus, the level of TIMP expression may be normal, but post-transcriptional or translational factors may affect TIMP maturity, stability, and/or availability. TIMP is more soluble in AAA tissue, therefore, the deficiency of TIMP may be due to increased degradation. TIMP is stable at extremes of temperature and pH [81]. It is possible that factors leading to decreased heat stability of TIMP in aneurysm patients could result in inactivation, and loss of its inhibitory action. Kontusaari [58] showed that a mutation leading to synthesis of a less heat stable procollagen resulted in aneurysm formation in a family. Mature TIMP has several N-linked glycosylation sites which are important for function [82]. Deficient or defective glycosylation in



aneurysm patients could result in decreased inhibition of metalloproteases. Further studies of the TIMP gene product from patients with AAA may be useful in resolving these issues.

The etiology of AAA is most likely multifactorial with the increased propensity for AAA in the infrarenal aorta due to biomechanical/physiological factors. Some aortic aneurysms are due to defects in structural proteins whether as a result of mutations in the structural genes themselves [58], or in the pathway leading to formation and turnover of structural proteins, as in the Blotchy mutation [38-42]. Elastase specific to AAA tissue is inhibited by copper, and may have increased activity in states of copper deficiency, which may be genetically controlled. Specific gene markers on chromosome 16 are associated with AAA [59]. Certain haptoglobin phenotypes have been found to accelerate elastolysis by two- to four-fold *in vitro*. It is possible that haptoglobin makes elastin more susceptible to degradation by binding to it, or there may be a gene on chromosome 16 that is associated with AAA, and in linkage equilibrium with the haptoglobin gene. These findings can explain proteolysis and the familial tendency for AAA, but does not account for the apparent male preponderance among patients with AAA, therefore, this may only account for some AAA.

The genetic basis of AAA formation is still to be determined. It is hoped that ultimately a specific gene abnormality or abnormalities will be identified to permit earlier detection of individuals predisposed to AAA formation, even prior to AAA development. Screening tests could then be developed to 1. detect previously unknown AAA for resection or observation, 2. permit more accurate

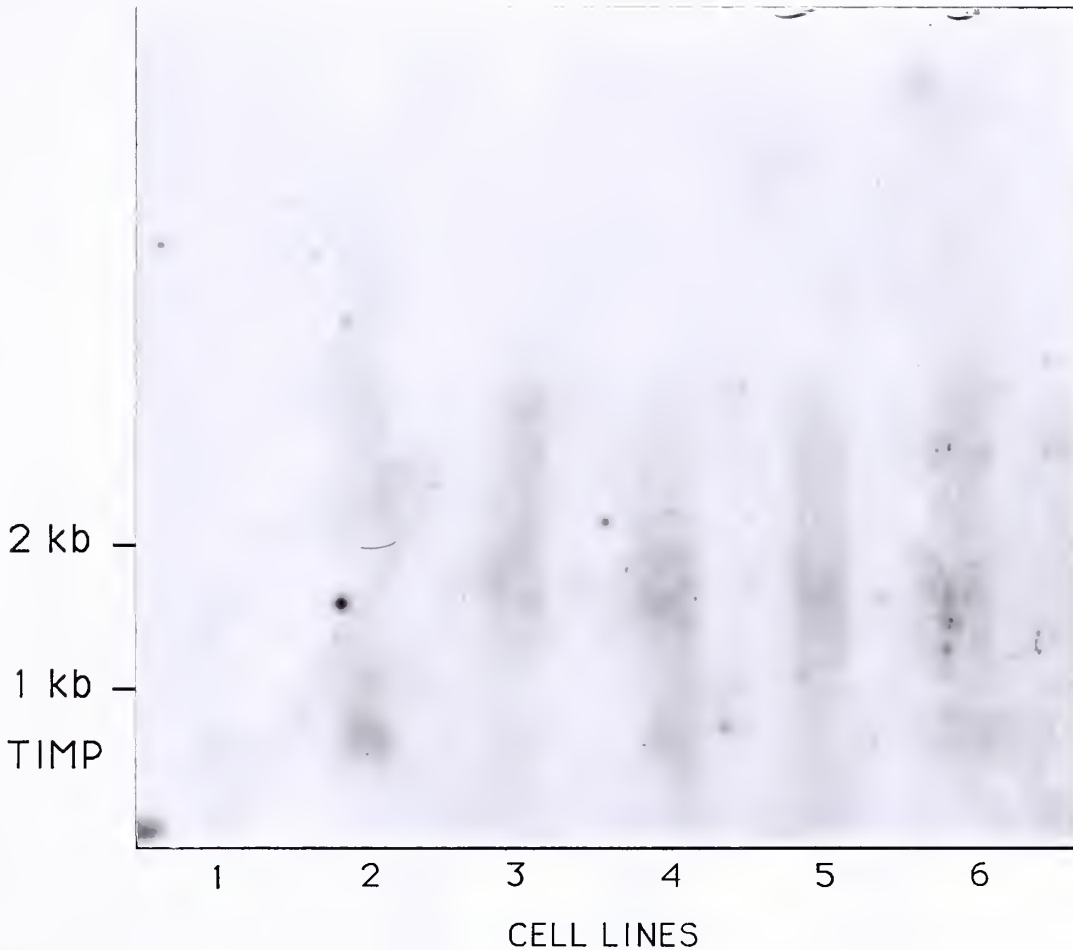


identification of affected and unaffected individuals in pedigrees to allow a more precise definition of mode of inheritance of susceptibility to AAA formation so that linkage studies to identify specific genes involved may be undertaken, and 3. identify a large enough pool of patients with small AAAs so that the natural history of AAA development can be studied. This would allow investigators to determine factors which contribute to enlargement, and permit randomized trials of drug therapy to retard or prevent AAA growth.

The genetic predisposition for AAA development is exacerbated by environmental risk factors such as hypertension, smoking, and diet. Patients with AAA should have their hypertension controlled, and should be encouraged not to smoke.  $\beta$ -adrenergic blockade has been shown to delay the formation of aortic aneurysms in Blotchy mouse [83] and humans [84], and to decrease the chance of rupture in turkeys with aneurysmal disease [13,14,45]. Propanolol inhibits development of aneurysms by a direct effect on collagen cross-linking independent of its effects on heart rate and blood pressure [45]. Therefore, treatment with  $\beta$ -blockers may have a two-fold effect: controlling hypertension and slowing aneurysm formation. Calcium-channel blockade has also been suggested to slow the progression of the disease in patients with small AAA [85]. Patients with AAA disease and their first-degree relatives should be screened by abdominal ultrasound, at least annually, to monitor aortic dilatation. Aneurysm size greater than 5 cm correlates with a clinically significant risk of rupture, therefore, elective repair should be considered if there is rapid expansion or increase in size to 5 cm or greater [35,86].







**Figure 1A. TIMP Expression in Different Cell Types**

10  $\mu$ g of total RNA from rat fibroblasts (1,2), rat smooth muscle cells (3,4), and bovine aortic endothelial cells (5,6) were electrophoresed in an agarose gel containing formaldehyde, transferred to a nitrocellulose filter, and probed with mouse TIMP cDNA. Northern blot shows TIMP expression in different cell types, and the effect of TGF- $\beta$  on TIMP expression. (TIMP cDNA  $\approx$  0.7 kb).



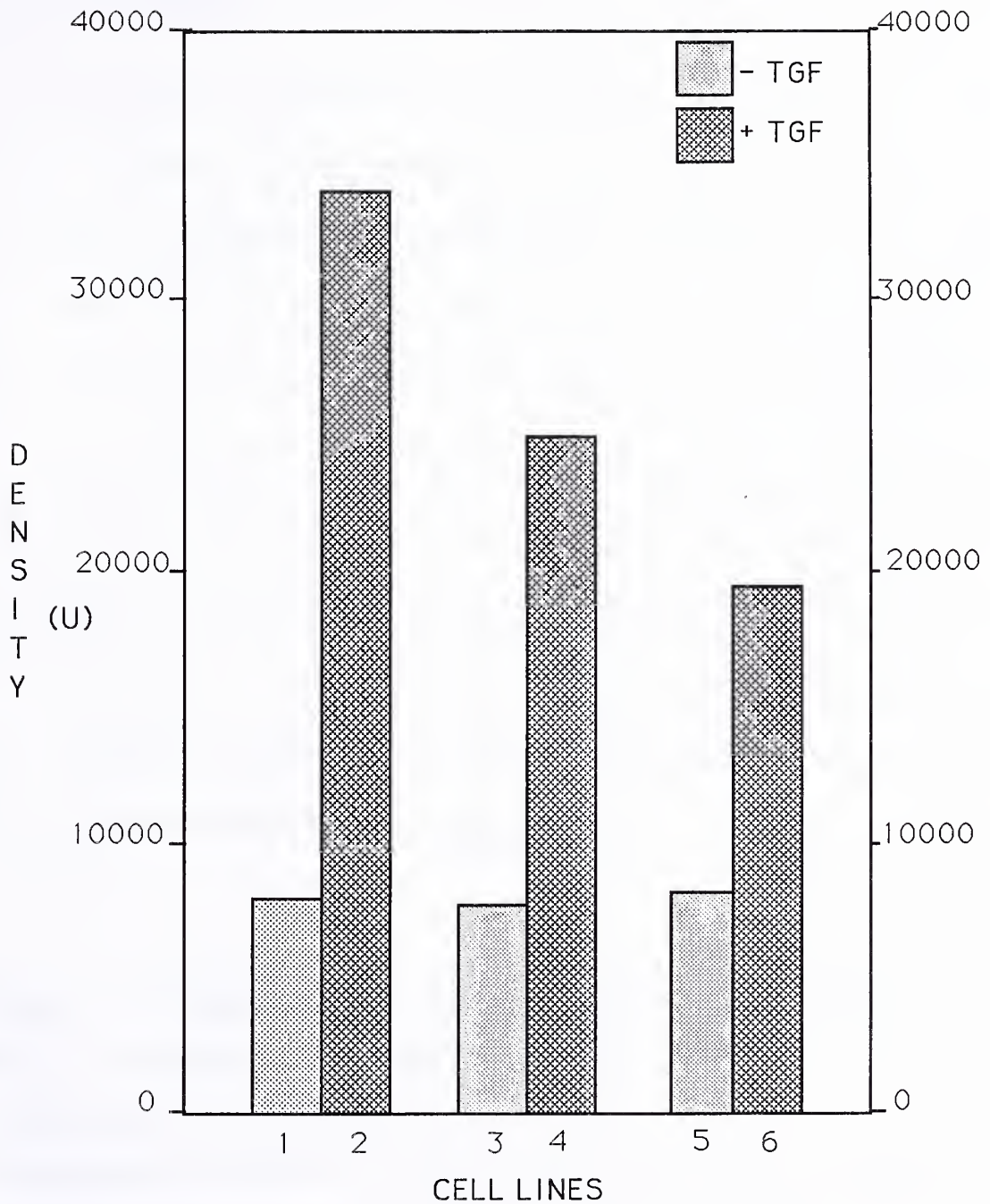


Figure 1B. TIMP Expression in Different Cell Types

Densitometry was performed on the northern blot shown in figure 1A to evaluate the effect of TGF- $\beta$  on TIMP expression in rat fibroblasts (1,2), rat smooth muscle cells (3,4), and bovine aortic endothelial cells (5,6).

(U = arbitrary densitometry units)







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1  agcgcccagagagacaccagagaaccaccatggccccctttgagcccctggcttctggc 60
      N A P F E P L A S G
61  atcctgttggtgctgtggctgatageccccagcagggcctgcacctgtgteccaccac 120
      I L L L L W L I A P S R A C T C V P P H
121  ccacagacggccttctgcaattccgacctcgtcatcagggccaagtctggtgggacacca 180
      P Q T A F C N S D L V I R A K F V G T P
181  gaagtcaaccagaccaccttataccagcgttatgagatcaagatgaccaagatgtataaa 240
      E V N Q T T L Y Q R Y E I K M T K M Y R
241  gggttccaagccttaggggatgccgctgacatccggttcgtctacacccccgccatggag 300
      G F Q A L G D A A D I R F V Y T P A M E
301  agtgtctgctggatacttccacaggtcccacaaccgcagcagaggagtttctcattgctgga 360
      S V C G Y F H R S H N R S E E F L I A G
361  aaactgcaggatggactcttgcacatcactacctgcagtttcgtggctccctggaacagc 420
      K L Q D G L L H I T T C S F V A P W N S
421  ctgagcttagctcagcgcggggcttcaccaagacctacactgttggctgtgagggaatgc 480
      L S L A Q R R G F T K T Y T V G C E E C
481  acagtgtttccctgtttatccatcccctgcaaactgcagagtgggcactcattgcttgtgg 540
      T V F P C L S I P C K L Q S G T H C L W
541  acggaccagctcctccaaggetctgaaaagggttccagtcaccgtcaccttgccctgctg 600
      T D Q L L Q G S E R G F Q S R H L A C L
601  cctcgggagccagggctgtgcaacctggcagtcctcgggtcccagatagcctgaatcctg 660
      P R E P G L C T W Q S L R S Q I A
661  cccggagtggaagctgaagcctgcacagtgctccacc 697

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### Figure 2B. Sequence of TIMP cDNA from AAA Patient

PCR products from TIMP cDNA primed with oligos TIMP1 & TIMP3, or with TIMP2 & TIMP3 were ligated with BamHI-cleaved Bluescript KS+ plasmid vector DNA. Sequencing was performed in both directions directly from double-stranded DNA by the dideoxynucleotide method of Sanger *et al.* The sequence is identical in nucleotide and amino acid sequence to that published by Docherty *et al* as the normal sequence.





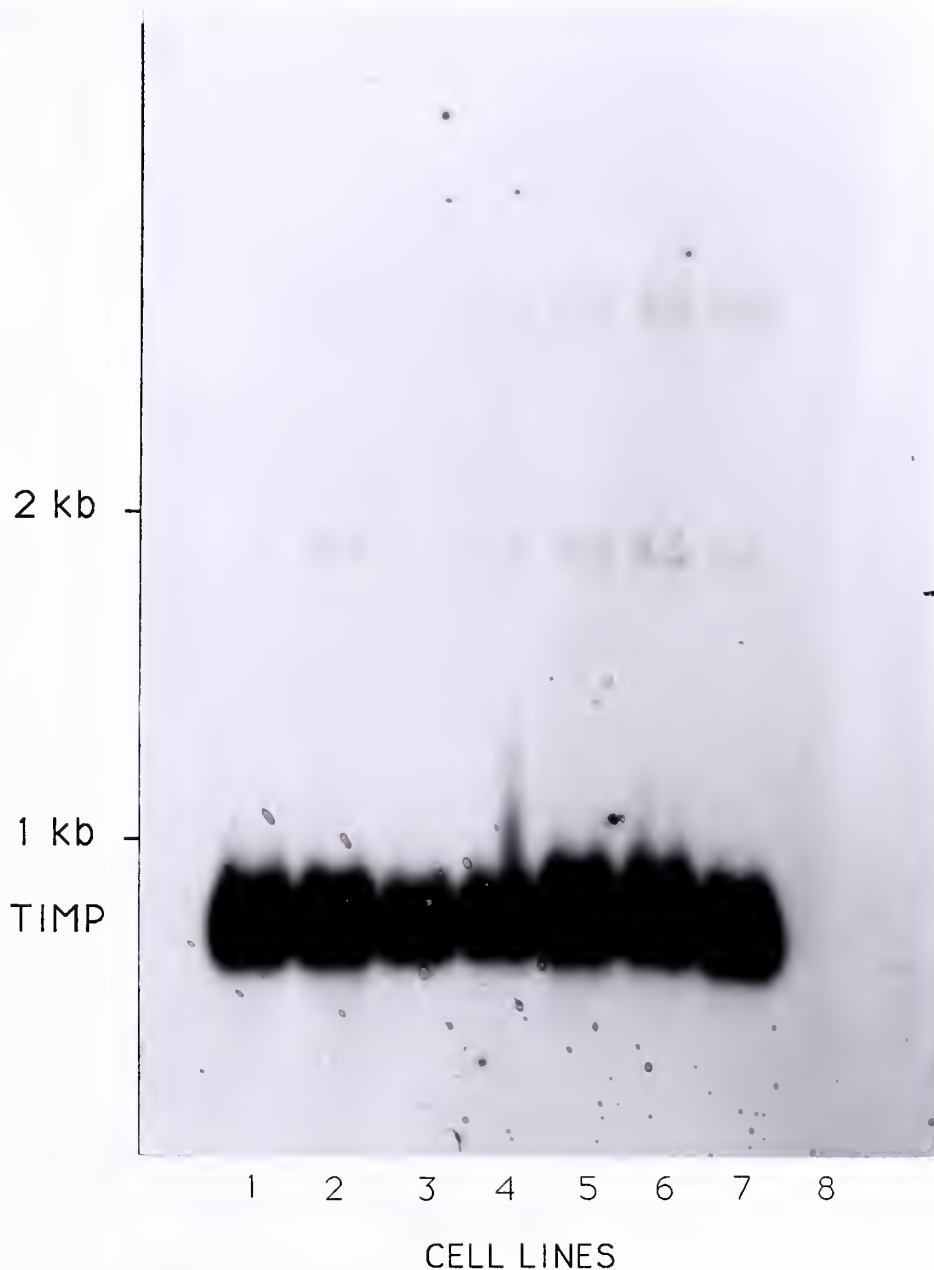


Figure 3A. TIMP Expression in AAA

15  $\mu$ g of total dermal fibroblast RNA from six patients with AAA (1,2,3,4,6,7) and one patient with occlusive disease (5), and 15  $\mu$ g of total lymphoblast RNA (8), were electrophoresed, transferred to a nitrocellulose filter, and probed with human TIMP cDNA. Northern blot shows TIMP expression in AAA and nonAAA dermal fibroblasts. TIMP is not expressed in the lymphoblast cell line. (TIMP cDNA  $\approx$  0.7 kb).



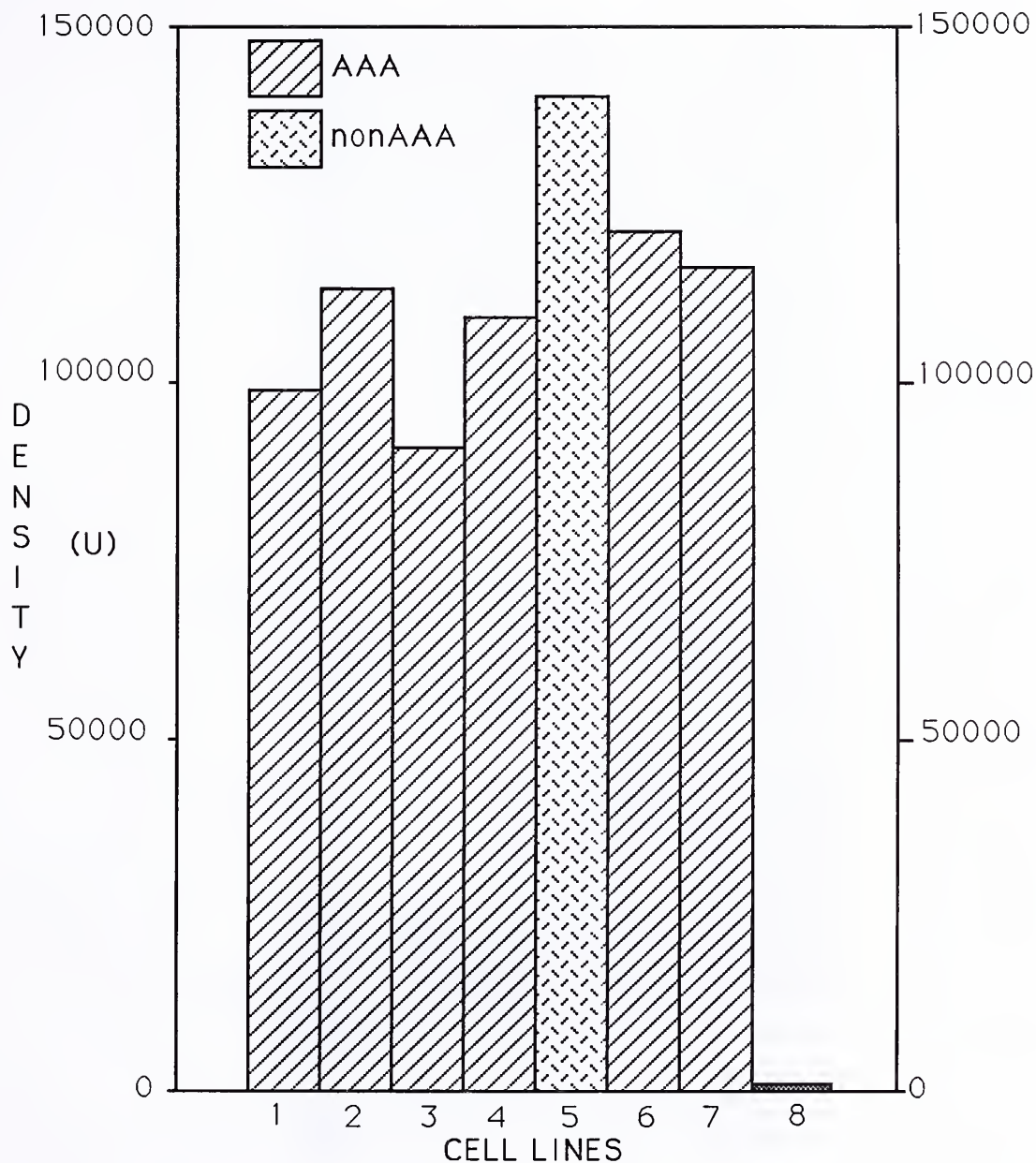


Figure 3B. TIMP Expression in AAA

Densitometry was performed on the northern blot shown in figure 3A, to compare TIMP expression in AAA patients (1,2,3,4,6,7) with a nonAAA control (5). Cell line #8 is a non-TIMP expressing control. (U = arbitrary densitometry units)



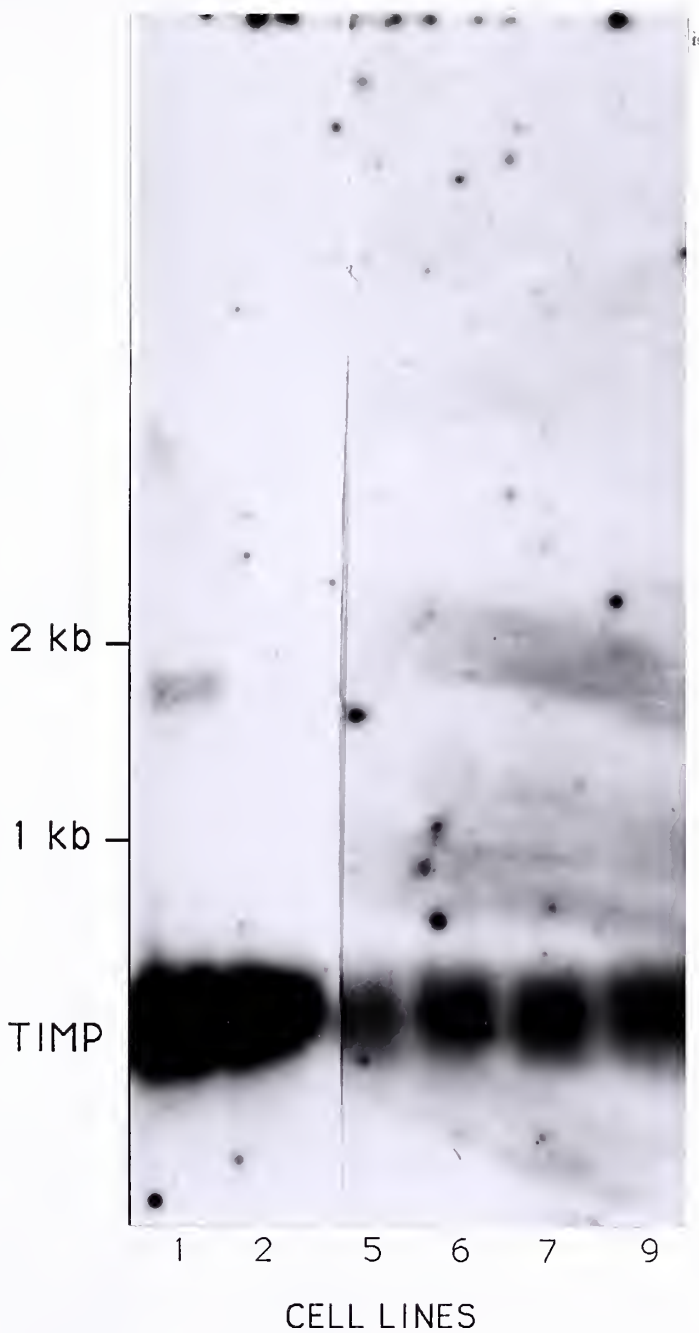


Figure 4A. TIMP Expression in AAA

10  $\mu$ g of total dermal fibroblast RNA from four patients with AAA (1,2,6,7), one patient with occlusive disease (5), and one normal patient (9) were electrophoresed, transferred to a nitrocellulose filter, and probed with human TIMP cDNA. Northern blot shows TIMP expression in AAA and nonAAA dermal fibroblasts. (TIMP cDNA  $\approx$  0.7 kb).



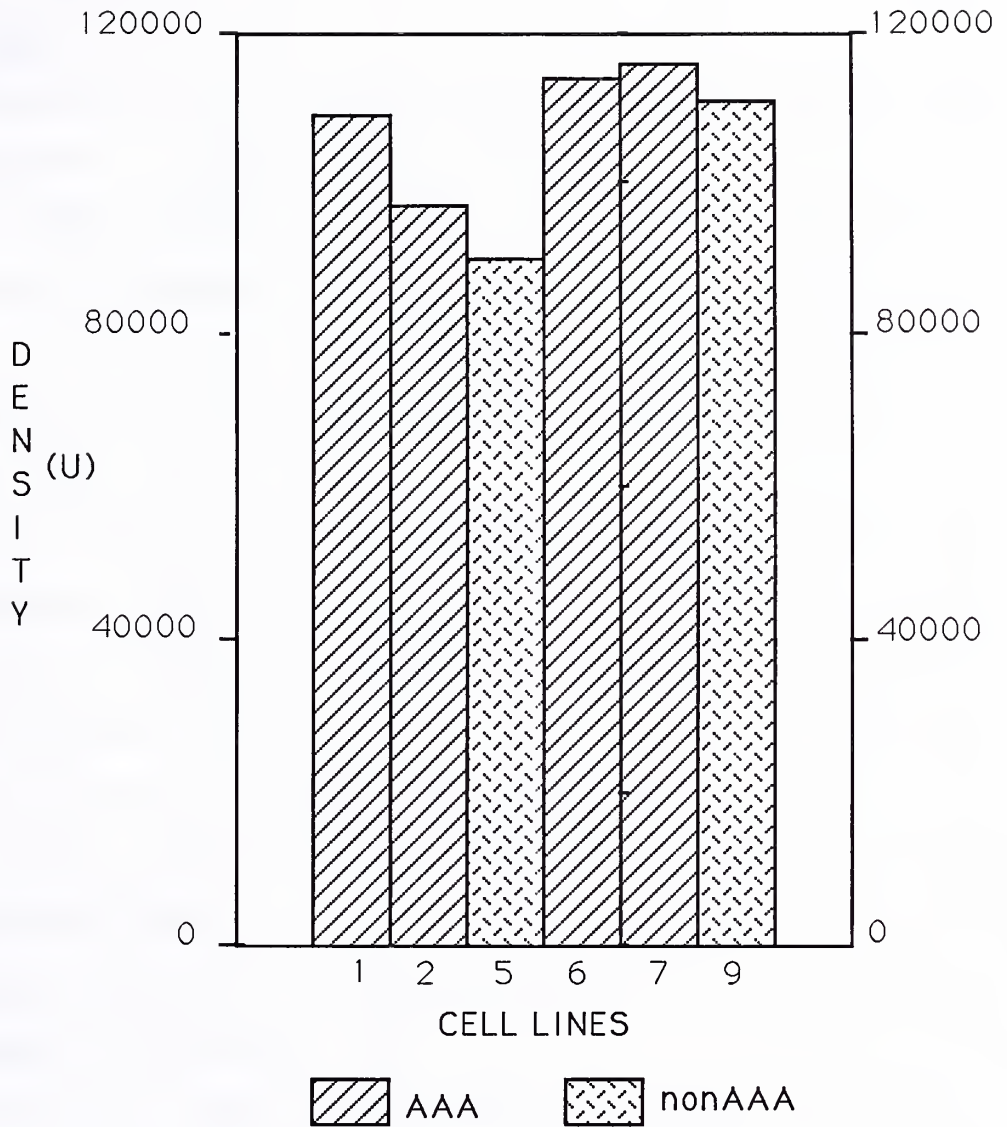


Figure 4B. TIMP Expression in AAA

Densitometry was performed on the northern blot shown in figure 4A, to compare TIMP expression in AAA patients (1,2,6,7), with two nonAAA controls: a patient with occlusive disease (5), and a normal patient (9). (U = arbitrary densitometry units)





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