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Role of TGF beta signaling in Remodeling of Non-Coronary Artery Aneurysms in Kawasaki disease

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Aaron Ming Lee

Committee in charge:

Professor Jane C. Burns, Chair
Professor Li-Fan Lu, Co-chair
Professor Laurie G. Smith

2014
The Thesis of Aaron Ming Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

University of California, San Diego

2014
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LIST OF ABBREVIATIONS

KD- Kawasaki Disease

CAA- Coronary Artery Aneurysm(s)

NCAA- Non-coronary Artery Aneurysm(s)

TGFβ- Transforming Growth Factor beta

WSS- Wall Shear Stress
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I would like to acknowledge Dr. Jane C. Burns for her invaluable role in my work, both as my chair and my mentor. If she had not shown me the bridge between the clinic and laboratory, I would not be writing this thesis. She has always hoped to inspire her students; she has certainly inspired me.

I would also like to acknowledge Dr. Chisato Shimizu, whose constant patience and continued guidance made this entire project possible. Her dedication in lab and unwavering mentorship have shown me that passion in for good work in the laboratory can truly lead to great results.

I would finally like to acknowledge the entire Kawasaki Disease Research Center team at UCSD, whose prior work is the basis for the data presented here, and whose continued work will change the lives of countless children. This study would not have been possible without their support. I will forever be in debt to the KD Research Center for allowing me to join their family.
ABSTRACT OF THE THESIS

Role of TGF beta signaling in Remodeling of Non-Coronary Artery Aneurysms in Kawasaki disease

by

Aaron Ming Lee

Master of Science in Biology

University of California, San Diego, 2014

Professor Jane C. Burns, Chair
Professor Li-Fan Lu, Co-Chair

Coronary artery aneurysms remain a life-threatening complication of Kawasaki disease (KD), the most common form of pediatric acquired heart disease in developed countries (1). Potentially life-threatening coronary artery aneurysms (CAA) develop in 25% of untreated children and 5% of children treated with high dose intravenous immunoglobulin during the acute phase of the self-limited vasculitis (2). Non-coronary artery aneurysms (NCAA) in extra-parenchymal, muscular arteries occur in a minority of patients with KD (2%) who also develop coronary artery aneurysms (CAA) (3, 4), yet little is understood in their formation and remodeling. We postulated that activation of the transforming growth factor TGFβ pathway may influence formation and remodeling of
aneurysms in iliac and axillary arteries in KD, the two most common sites for NCAA. We studied the resected axillary artery from one adult and endarterectomy tissue from the iliac artery from a second adult, both with a history of CAA and NCAA following KD in infancy. Histology revealed destruction of the internal elastic lamina with myofibroblastic luminal proliferation, organized thrombus, and recanalization of the axillary artery aneurysm and organized thrombus with dense calcification in the endarterectomy specimen. Immunohistochemistry for molecules in the TGFβ signaling pathway revealed increased expression of TGFβ2, TGFβ receptor, and phosphorylated SMAD3. These findings suggest on-going tissue remodeling of the aneurysms decades after the acute injury and demonstrate the importance of the TGFβ signaling pathway in this process.
INTRODUCTION

Kawasaki disease (KD) was first identified in 1967 by Dr. Tomisaku Kawasaki in Tokyo, Japan when he published his description of 50 children all showing distinct clinical signs and symptoms that were otherwise uncategorized into any other disease. Through meticulous tracking of each child’s symptoms, Dr. Kawasaki identified several clinically important criteria that are still used today to make a diagnosis of KD. A fever of more than 4 days is a requirement for diagnosis, and must be found with at least four of the following symptoms: bilateral conjunctival injection (red eyes), mucous membrane changes (cracked lips, strawberry tongue), changes of peripheral extremities (swollen hands/feet, red palms/soles), polymorphous rash, or cervical lymphadenopathy (swollen lymph nodes) (Figure 1). An additional clinical criterion for KD allows for diagnosis if the patient has fever for more than 5 days, has fewer than 4 of the above clinical features, and yet has echocardiographic evidence of coronary artery disease (5).

KD is an acute, self-limited vasculitis, and remains the leading cause of acquired heart disease in children in developed countries. In Japan, an average of 138 children under the age of 5 years in every 100,000 are diagnosed with KD, while in the U.S., 22 in every 100,000 children under the age of 5 are diagnosed (6). As a pediatric disease, KD is found mostly in children under the age of 5. In the U.S. between 1997 and 2000, children less than 5 years of age made up 77% of KD hospitalizations (7).
Figure 1: Clinical features of KD
Symptoms listed clockwise from top left: Bilateral conjunctival injection, polymorphous rash, changes of peripheral extremities, cervical lymphadenopathy, mucous membrane changes.

A significantly increased incidence among Asian/Pacific Islanders suggests a genetic influence on KD susceptibility. Genome-wide association studies have shown that there are a number of genetic polymorphisms that may be associated with KD susceptibility (8). KD pedigrees have also shown the potential for multiple affected members within a family tree, though the hereditary patterns are not definitive (9).
The etiology of the disease remains unknown, though evidence suggests that a foreign agent is responsible for triggering the disease in individuals who are genetically susceptible. Recently, evidence has been shown that suggests that a region in northeastern China serves as the source of the wind-borne etiologic agent of KD. Samples taken over Japan showed significant differences in the microbiota in the tropospheric aerosol as compared to the ground aerosol. Wind patterns consistent with seasonal outbreaks of KD further enforce the growing indication that KD is an immune response to a currently unknown wind-borne antigen (10).

KD is self-limited; the symptoms will develop over the first 10 days of illness but will resolve spontaneously, even without targeted treatment. However, coronary artery aneurysms (CAA) remain a life-threatening complication of KD, and are often the target of concern when patients present with KD. CAA are often clinically silent and may remain unnoticed until several years later. These CAA will form in 25% of untreated children. Treatment with high dose intravenous immunoglobulin (IVIG) during the acute phase lowers this rate to 5%. Nonetheless, a timely diagnosis is important in KD such that children who would benefit from treatment with IVIG can be identified (11).

The epicardial coronary arteries are the most common site of aneurysm formation in patients with KD, although aneurysms may also develop in the non-coronary arteries in a subset of patients with CAA. Early autopsy studies from Japan suggest that certain non-coronary artery locations are more susceptible to aneurysm and arteritis formation in KD, particularly the iliac and axillary arteries (12, 13). Medium-sized, muscular arteries
consistently show higher susceptibility to aneurysm formation in KD, and of these arteries, the iliac and axillary arteries show the highest incidence rate (12, 14).

By analogy, Marfan’s disease is a connective tissue disorder in which a mutation in *Fibrillin-1* leads to formation of aneurysms. Overactivation of the TGFβ signaling pathway has been shown to play a key role in the formation and remodeling of these aneurysms, both in human and mouse models (15). This led to the initial hypothesis that TGFβ is important in CAA in KD. A genetic study of correlation between 164 SNPs in 15 genes in the TGFβ pathway found three genes associated with increased risk of CAA in KD patients: TGFβ2, TGFβ Receptor (R)2, and SMAD3 (8). Further studies of the pathway through immunohistologic examination of autopsy tissues demonstrated the expression of TGFβ signaling molecules in the aneurysm wall and the presence of myofibroblasts secreting matrix metalloproteinases (MMPs) and IL-17 with the recruitment of inflammatory cells into the media of the artery (16).

Other studies further suggest a link between TGFβ and aneurysms in KD. Evidence supports a connection between low shear stress at branch points as a contributing factor in aneurysm formation by activating the transforming growth factor (TGF)β signaling pathway in endothelial cells in CAA in KD (17). We proposed that increased regional expression of TGF-β signaling pathway molecules is an early step in aneurysm formation in acute KD. We further postulated that this signaling pathway might also influence remodeling of aneurysms in iliac and axillary arteries in KD.
MATERIALS AND METHODS

Subjects and tissue samples

Cases 1 and 2 were a 31 year old male and 22 year old female both diagnosed with acute KD at age 1-2 months of age who developed coronary, iliac, and axillary artery aneurysms. Both patients were enrolled as subjects in the San Diego Adult KD Collaborative study and completed general and cardiovascular health questionnaires. Surgically resected tissues included an endarterectomy specimen from the iliac artery of Case 1 and a completely resected axillary artery aneurysm from Case 2. All protocols were approved by the Institutional Review Board at UCSD and written consent was obtained for the use of all tissues.

Histological examination

Surgical specimens were formalin-fixed and paraffin embedded following surgical resection. 5 μM sections were made. Histochemical staining with hematoxylin-eosin (H&E), Verhoeff-van Gieson, and Masson’s trichrome stains was performed using standard techniques.

Immunohistochemical staining

Tissue sections were baked at 65°C for 60 minutes prior to deparaffinization and rehydration using standard techniques. Endogenous peroxidase activity was quenched using Peroxidase Block (LSAB+System-HRP, DAKO) for 10 minutes. Antigen retrieval was performed in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) in a microwave oven for 10 minutes. Slides were incubated in blocking solution
consisting of 5% normal swine serum (Jackson ImmunoResearch Swine Serum) in 0.01M Tris-HCl with 1% BSA for 60 minutes at room temperature. Slides were then incubated overnight at 4°C with the following primary antibodies diluted in 0.01M Tris-HCl with 1% BSA: anti-TGFβ2 (sc-90, SantaCruz, 1:100 dilution), anti-TGFβR2 (ab-78419, Abcam, 1:100 dilution), anti-pSMAD3 (ab-52903, Abcam, 1:250 dilution). Antibodies were detected using a biotin-streptavidin immunoperoxidase method (LSAB+System-HRP, DAKO) followed by visualization with the AEC substrate kit (Vector) according to manufacturer’s instructions. Normal rabbit immunoglobulin G (Jackson ImmunoResearch, 1:10,000 dilution) was used as a negative staining control in each run. Nuclear staining was performed using 1:2 diluted hematoxylin.

**Review of published KD peripheral aneurysms cases**

To better understand the frequency and presentation of peripheral artery aneurysms in KD, a comprehensive review of the English language literature was performed using PubMed with search keywords “Kawasaki Disease”, “iliac”, “axillary”, “aneurysm”, and “pathology”. Coronary artery status, age of onset, sex, and ethnicity were recorded for all cases.
RESULTS

Case 1, a 31 year old white male, first developed acute KD at age 7 weeks, though the diagnosis was initially missed until confirmed later by echocardiography, which showed large coronary artery aneurysms in both the right (RCA) and left anterior descending (LAD) coronary arteries and the left main coronary artery (LMCA). The patient was also noted to have bilateral axillary aneurysms. He was treated with dipyridamole and aspirin as this was prior to advent of intravenous immunoglobulin (IVIG) therapy. At age 22 he developed right calf claudication. No obvious aneurysms were found in the iliac arteries, though an irregularity in the proximal right iliac was noted for 2 cm. The calf claudication returned at age 25 and a lower extremity angiogram showed 50-60% stenosis in proximal right external iliac artery and tandem 50% stenosis in left iliac and popliteal artery. No intervention was undertaken and at age 30 years, the patient sought participation in the Adult KD Collaborative Study at UCSD/San Diego Cardiac Center. A medical history revealed no risk factors for atherosclerotic disease. Physical examination revealed a BMI of 26.04, blood pressure 124/80, heart rate 75/min. Cardiac catheterization demonstrated a 6-7mm distal right coronary artery aneurysm, a 3 mm aneurysm in the circumflex, and complete occlusion of the LAD. Lower extremity angiography now revealed severe disease in both iliac arteries with 90% occlusion of the right iliac artery, 99% stenosis of the right iliac ostium, and 70% narrowing distal to the ostium with a 95% stenosis of mid-portion of the artery. The patient underwent a left iliac endarterectomy (Figure 2A) and, in a second procedure, a right iliac artery endarterectomy with stent placement.
Figure 2: Endarterectomy and histological findings of left iliac artery aneurysm in Case 1

A. Left iliac artery with thrombus was exposed and cut incision longitudinally. Inside of the artery was filled with long thrombus (white arrow). B. Low magnification of resected tissue (50x, H&E). A large area of calcification (black arrow), partially decalcified, is visible in intimal tissue. Lumen (L) is indicated. Box labeled C-D is shown in higher magnification in subsequent images. C. Higher magnification (400x, H&E) of boxed area in panel B. Internal elastic lamina (black arrows) is followed. Intimal thickening (i) is visible. (400x, H&E) D. Elastic wall is clearly visible along outer edge of sample (white arrows) (400x, elastin) E. Organized thrombus removed from inside of arterial wall. Large area of calcification (black arrow) has been almost entirely decalcified. Box labeled F is shown in higher magnification in subsequent image (50x, H&E) F. Possible neovascularization with endothelial-like cells in lumen. (black arrow). (400x, H&E)
Intimal tissue removed during left iliac endarterectomy was stained with H&E (Figure 2B) revealing a thickened intima (Figure 2C) with diffuse fibrosis is detected. An intact elastic lamina (Figure 2D, arrows) was observed in the intimal layer. At higher magnification, many spindle shaped cells were noted along the luminal side of intima. The organized thrombus (Figure 2E) contained a large area of calcification that was lost during processing (black arrow). A possible area of neovascularization in the thrombus was noted (Figure 2F) with endothelial-like cells lining the lumen (arrow).

Figure 3: Histological findings of right iliac artery aneurysm resected by endarterectomy A and D. Low magnification of resected tissues (20x, 30x H&E). Boxes denote areas shown in higher magnification in subsequent images. B. Higher magnification of boxed area in panel A (200x, H&E). Thickened intima (i), newly formed elastic lamina (white arrow) are visible. Foam cells (blue circle) C. Internal elastic lamina (white arrow) is clearly visible along edge of sample (200x, elastin). E. Higher magnification of boxed area in panel D (100x, H&E). Internal elastic lamina is attached to this portion of tissue (black arrow). Thickened intima (i), internal elastic lamina (black arrow) and plaque (p) are visible in section. F. Diffusely fibrotic areas (blue) are visible in intima (i) layer. (100x, Trichrome).
The right iliac endarterectomy specimen contained two intimal layers with internal elastic laminae (Figure 3A,D) and an organized thrombosis (not shown). The inner intima layer (Figure 3A) was thickened (Figure 3B), and contained a newly formed elastic lamina (Figure 3B,C). Foam cell accumulation was noted along the elastic lamina and near a small area of calcification (Figure 3B). No other changes of atherosclerosis were noted. The inner intimal layer (Figure 3D) was thickened intima (Figure 3E) and diffusely fibrotic (Figure 3F).

Case 2 was a 21 year old white female who developed KD at age 1 month, but the diagnosis was initially missed. She was officially diagnosed at age 4 months, at which time she was noted to have giant coronary aneurysms by echocardiography. She was treated with aspirin. At age 18 months, she had a second episode of KD, at which time she was noted to also have bilateral axillary aneurysms. The coronary artery aneurysms remodeled, though the bilateral axillary aneurysms remained persistent. At age 19 she underwent a left axillary artery bypass surgery for symptoms of left arm claudication. At age 21, she enrolled in the San Diego Adult KD Collaborative study. Her chief complaint was chest pain and exercise intolerance.

She underwent coronary angiography with intravascular ultrasound that revealed a discrete right axillary aneurysm with 80-90% stenosis. She underwent right axillary bypass surgery.
Figure 4: Histological findings of right axillary artery aneurysm in Case 2

A. Low magnification of resected right axillary artery. “Lotus root” arterial conformation is clearly visible, as well as large pockets of calcification (dark purple). Box denotes area shown in higher magnification in subsequent images. B. Higher magnification (400x, H&E) of boxed area in panel A. Endothelial cells (black arrows) are noted along lumen. C. Elastic wall (black arrows) is clearly visible. (400x, Elastin).

The resected axillary artery aneurysm showed diffuse changes in all layers of the arterial wall. A classic “lotus root” conformation was noted with recanalization and neovascularization in multiple channels of the completely occluded artery (Figure 4B). Several lumens are indicated (L). Large pockets of calcification were seen (purple). Heavy deposits of collagen, suggestive of diffuse fibrosis, were evident through several layers of artery (not shown). However, there were no changes consistent with atherosclerosis. Endothelial-like cells are clearly seen along lumen edge (Figure 4B). Original elastic lamina has been destroyed, though a new lamina (Figure 4C) can be clearly seen along recanalized lumen.
Aneurysms from Case 1 and Case 2 were examined for evidence of TGFβ pathway activity. Immunohistochemistry was done in order to visualize TGFβ pathway markers of activation. Positive staining for the receptor, TGFβ2, was observed in spindle shaped cells in the thickened intima of the arteries, but not in control tissues. TGFβ2 is expected to be constitutively expressed in all samples, and localized to the cytoplasm of cells. Staining for the TGFβ2 ligand resulted in cell expression throughout the intimal layers presented. TGFβR2 is localized to the membrane, as well as some localization in
the cytoplasm. All aneurysm samples expressed TGFβR2. Further staining was done to
detect nuclear localization of activated SMAD3. Phosphorylated SMAD 3 (pSMAD3) is
a downstream marker of TGFβ pathway activation. There were variable results in
detection of pSMAD3 in the arteries of Case 1 and Case 2. Staining was detected in
nuclei of the left iliac and right axillary arteries, and some staining was noted in the right
iliac artery as well, though considerably less than the other arteries. Control staining with
Rabbit IgG showed no staining in any of the tissue samples.

By comprehensive study of published English Language literature, 20 KD cases
were found (Table 1) in which the patient developed aneurysms in the iliac and/or
axillary arteries. Interestingly, out of 20 KD patients found, 75 % had an age of onset of
less than or equal to 6 months of age. 6 patients had aneurysms in both the axillary and
iliac arteries.
Table 1: Review of published cases of KD patients who developed iliac and/or axillary aneurysm

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DISCUSSION

Iliac and axillary artery aneurysms show similar remodeling pattern to CAA in KD

The evidence presented here suggests that the TGFβ signaling pathway plays an important role in the remodeling of non-coronary artery aneurysms in KD, similar to the role the TGFβ pathway plays in CAA in KD. Through histological studies, we have shown that the aneurysms of the axillary and iliac arteries share similar structural changes to those seen in CAA in KD. These changes include destruction of the internal elastic lamina, thrombus formation, and inflammation (16, 21). Additionally, diffuse fibrotic material and newly formed elastic laminal walls along recanalized arteries were seen, with spindle-shaped cells lining the lumen. We also demonstrated expression of several TGFβ pathway gene products in the axillary and iliac arterial walls. These results suggest remodeling of non-coronary artery aneurysms through the TGFβ pathway.

Outside of CAA, iliac and axillary artery aneurysms show prevalence

Medium-sized muscular arteries are particularly prone to aneurysm formation in KD (14). Outside of the coronary arteries, the iliac and axillary arteries consistently show higher susceptibility to aneurysm formation in KD, with 2% of patients forming aneurysms (3). The iliac and axillary arteries have been identified as the most susceptible peripheral arteries to form aneurysms in KD (12).
Several factors contribute to site specificity of aneurysm formation in iliac/axillary

1) The effects of changes in fetal blood circulation, repetitive positional compression

Higher prevalence of aneurysm formation in the axillary and iliac arteries in KD suggests that there are factors contributing to aneurysm susceptibility in these locations. Evidence suggests that the tendency of aneurysms to form in certain locations is determined by structural characteristics. The iliac arteries display a shift in blood flow between fetal and mature stages. Fetal iliac arteries direct blood towards the internal iliac arteries, which carry a larger load of blood in comparison to the external iliac arteries. This can lead to “wear and tear” of the internal iliac arteries. However, upon birth, the infant diverts the blood to the external iliac arteries, as the blood flow needs have shifted away from fetal circulation and more towards the extremities. The shift in mechanical load may lead to structural changes in the arterial integrity, leading to weakening of the arterial wall. Weakened arterial walls may predispose a particular infant to formation of aneurysms in the iliac artery (26), especially given the systemic inflammation in KD. Axillary artery aneurysms have been shown to also form via repetitive positional compression, in which the humeral bone compresses the axillary artery repeatedly. This repeated stress may lead to weakening of the arterial wall, which in turn may lead to aneurysm formation (27, 28). While this has been largely shown in professional baseball pitchers and overhead throwing athletes, it is possible that young children who repeated rotate their arms above their heads may produce similar structural stress on their developing axillary arteries.
2) Wall Shear Stress (WSS)

Studies of aneurysm site specificity suggest that there are a number of other hemodynamic and structural factors that play important roles in directing aneurysm formation, including branching characteristics of the artery, size of the artery, and shear stress (29). A structural issue that may play an important role in the development of aneurysms in these particular regions of the cardiovascular system involves the concept of wall shear stress (WSS). Shear stress is the force a fluid applies to its containing structure as the fluid passes through. Low velocity occurs in areas of large diameter and leads to lower shear stress, while high velocity areas with laminar flow lead to higher shear stress (30). Variant amounts of WSS can lead to targeted expression of genes and gene groups, and thereby lead to specific gene expression in different arterial areas (31). Branch points between arteries tend to have areas of lowered shear stress, and these same areas have a significantly higher prevalence of aneurysm formation. (29). WSS has previously been shown to play a role in directing formation of aneurysms in CAA in KD. In an analysis of 111 KD patients, 90.3% if CAA formed at branch sites, and branch sites were noted to have significantly low WSS (17). Significantly lowered WSS in CAA in KD has also been shown to be correlated with thrombus formation due to increases particle residence time in the dilated region of the coronary artery (32). The iliac and axillary arteries present areas of interest in studying WSS, namely in their potential for predisposition towards having areas of lowered WSS. The large branch point in the iliac artery is the most common site for aneurysm formation, and these areas are directly correlated with areas of significantly lowered WSS (29). The axillary artery also shows branching into several arteries, including the superior thoracic artery, thoracoacromial
artery, lateral thoracic artery, subscapular artery, anterior circumflex humeral artery, and posterior circumflex humeral artery (33). These branch points provide areas of turbulent blood flow and lowered WSS, leading to potential aneurysm formation.

WSS is known to also trigger a number of genes (29), one of which is TGFβ (34). TGFβ has been shown to play important roles in aneurysm formation in other disease models, most notably Marfan’s syndrome and Loeys-Dietz syndrome. In these models, overactivity of TGFβ was found to play an important role in aneurysm formation (15), and mutations affecting TGFβ receptors have been found to predispose Loeys-Dietz patients to vascular disease (35). Previous studies have shown that coronary artery aneurysms in KD are influenced by TGFβ activity as well (16) and that KD patients may have a genetic predisposition towards increased activity by TGFβ (8). TGFβ is known for playing a role in the inflammatory response, recruiting myofibroblasts, facilitate endothelial-to-mesenchymal transition into endothelial layers, and increase migration of cells (8). Overactivity of TGFβ in the iliac and axillary arteries aneurysms in KD suggest a similarly important role for TGFβ in the remodeling of peripheral arteries. Significantly lowered WSS, along with other structural and genetic factors, may lead to overactivity of TGFβ in susceptible areas in the iliac and axillary arteries, leading an increase in the weakening of the arterial wall.

**Strengths and limitations of study**

The strength of this study is in its ability to have complete clinical data and patient history to supplement the research work. The Kawasaki Disease Research Center
is able to provide a comprehensive approach to the pathological results presented here. Samples presented in this study are generally difficult to obtain, and the specific non-coronary artery aneurysm data shown here is novel within the scope of Kawasaki disease. Nonetheless, this study is limited in its breadth, as only two patients are presented. A larger sample size would be desirable; however, tissues from KD patients with peripheral aneurysms are generally difficult to procure. Additionally, we have defined and described myofibroblasts in solely an estimative fashion. Due to lack of access to equipment and appropriate samples, no electron microscopy work was done to identify the ultrastructural characteristics of myofibroblasts to determine if the cells identified are truly myofibroblasts.
CONCLUSION

The TGFβ signaling pathway may influence remodeling in peripheral artery aneurysms in KD. Previous studies by our group showed that genetic variation in TGFβ2, TGFβR2 and SMAD3 is associated with coronary artery aneurysm formation in KD. Although KD patients ≤ 6 mos. of age comprised only 25/417 (6.0%) of our KD patients over a 5 year-period, they comprised 75% of the reported cases with peripheral artery aneurysms. Further study of genetic risk factors for development of KD at a very young age may be instructive. The TGFβ pathway may be a therapeutic target to interrupt the progression of arterial aneurysms and a clinical trial of atorvastatin, which blocks myofibroblast transformation, is in progress at Rady Children’s Hospital in San Diego.
REFERENCES


30. Paszkowiak JJ, Dardik A. Arterial wall shear stress: observations from the bench to the bedside. Vascular and endovascular surgery 2003; 37, 47-57.


