Age-Related Changes in Aortic Valve Hemostatic Protein Regulation

Liezl R. Balaoing, Allison D. Post, Huiwen Liu, Kyung Taeck Minn, K. Jane Grande-Allen

Objective—Although valvular endothelial cells have unique responses compared with vascular endothelial cells, valvular regulation of hemostasis is not well-understood. Heart valves remodel throughout a person’s lifetime, resulting in changes in extracellular matrix composition and tissue mechanical properties that may affect valvular endothelial cell hemostatic function. This work assessed valvular endothelial cell regulation of hemostasis in situ and in vitro as a function of specimen age.

Approach and Results—Porcine aortic valves were assigned to 1 of 3 age groups: Young (YNG) (6 weeks); Adult (ADT) (6 months); or Elderly (OLD) (2 years). Histological examination of valves showed that secreted thrombotic/antithrombotic proteins localize at the valve endothelium and tissue interior. Gene expression and immunostains for von Willebrand factor (VWF), tissue factor pathway inhibitor, and tissue plasminogen activator in YNG porcine aortic valve endothelial cells were higher than they were for OLD, whereas plasminogen activator inhibitor 1 levels in OLD were higher than those for YNG and ADT. Histamine-stimulated YNG porcine aortic valve endothelial cells released higher concentrations of VWF proteins than OLD, and the fractions of VWF-140 fragments was not different between age groups. A calcific aortic valve disease in vitro model using valvular interstitial cells confirmed that VWF in culture significantly increased valvular interstitial cell nodule formation and calcification.

Conclusions—Hemostatic protein regulation in aortic valve tissues and in valvular endothelial cells changes with age. The presence of VWF and other potential hemostatic proteins increase valvular interstitial cell calcification in vitro. Therefore, the increased capacity of elderly valves to sequester the hemostatic proteins, together with age-associated loss of extracellular matrix organization, warrants investigation into potential role of these proteins in the formation of calcific nodules. (Arterioscler Thromb Vasc Biol. 2014;34:72-80.)

Key Words: aging ■ aortic valve ■ endothelial cells ■ hemostasis

Semilunar valve diseases, particularly those affecting the aortic valve (AV), cause >60% of valve disease mortality, and ≈50,000 procedures to replace or repair semilunar valves are performed each year in the United States.1 Semilunar valve dysfunction affects all ages, from congenital valve defects experienced by neonates and children to the growing number of calcified valves in the elderly.1–6 These dysfunctional heart valves most often require surgical replacement using mechanical or bioprosthetic valves that may fail over time because of structural or thrombosis-related problems.6,7

To understand valvular disease and develop noninvasive therapeutic solutions, it is necessary to improve fundamental knowledge about the response of valve cells and extracellular matrix (ECM) to surrounding environments in various physiological states. The characterization of changes in valvular biology with respect to aging has become especially important as the occurrence of AV sclerosis and calcification continues to increase in the elderly population.4,5,8

Although most studies of valvular biology use AV tissues and cells from adults (either animal models or human specimens resected at surgery), it is clear that consideration of specimen age is necessary to evaluate age-specific conditions and pathologies. Previous studies from our research group and others have identified numerous age-related changes in the ECM composition and mechanical properties of the AV, as well as in the valve cell phenotypes.5,6–12 Considerable tissue remodeling and growth occurs in AV tissues before adulthood.4,11,13 During fetal development, AV tissues have not yet formed trilaminate ECM structures or high degree of elastin and collagen alignment that is apparent in adult valves.6,11,13

To mediate this microstructural organization of the mature AV, the developing AV also demonstrates cellular activation of both valvular interstitial cells and valvular endothelial cells (VECs).5,11,12

As subjects reach adulthood, valve cells become quiescent and maintain homeostasis of the highly organized valve leaflet structure and function.9 However, the AV continues to change with aging. Older valves have increased thickness and increased levels of collagen type III interspersed with proteoglycans and glycosaminoglycans throughout the
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observed to have strong interactions with valvular interstitial environments.19,21,22 These cells can undergo endo-
thelial to mesenchymal transdifferentiation, and thus play an
important role in valvulogenesis during fetal development.23,24

It is well-known that vascular endothelial cells perform sub-
stantial antithrombotic roles via production and release of tis-
sue plasminogen activator (tPA), tissue factor pathway inhibitor
(TFPI), von Willebrand factor (VWF) cleaving enzyme (a dis-
integrin and metalloprotease with a thrombospondin type I motif, member 13 [ADAMTS-13]), nitric oxide, and hepa-
rin.28–30 Endothelial cells are also responsible for the production
and release of thrombolytic protein mediators such as plasmino-
gen activator inhibitor 1 (PAI-1), tissue factor (TF), VWF, and
P-selectin.28–30 Under homeostatic conditions, vascular endo-
thelial cells constitutively express and produce both antithrom-
botic and thrombotic mediators to balance hemostatic effects
and prevent thrombus formation. However, blood coagulation
and thrombus formation are quickly initiated by endothelial
cells in response to mechanical damage, ECM degradation, and
disease.28–30 The production and regulation of these hemostatic
factors have not been well-characterized in VECs.

This study will evaluate the aging-related changes in anti-
thrombotic and thrombotic capacity of AV tissues and VECs.
We hypothesize that aging affects the VEC regulation of
hemostatic proteins and their interaction with valve ECM
components, resulting in an unbalanced regulation of anti-
thrombotic and thrombotic proteins within the tissues. Thus,
the evaluation of AV tissues and VEC function with respect
to antithrombotic and thrombotic functions in aging valves
will provide insight into the specific factors and conditions
that affect VEC hemostatic regulation. This information is
also relevant to the development of valve replacement devices
because the breakdown and thrombosis formation associated
with mechanical and decellularized valve implants have gen-
erated interest in understanding how the natural antithrom-
botic behavior of VECs can be integrated into age-specific
tissue-engineered heart valves. Overall, there is a critical need
to understand the hemostatic behavior of VECs.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Hemostatic Proteins Distribute Differently Within AV Tissues
Immunostaining showed that endothelial cell–mediated anti-
thrombotic and thrombotic proteins were present in each of
the differently aged AV leaflet groups (Figure 1). The pro-
teins were often localized with the endothelium at the edges
of each leaflet. Many of these hemostatic mediators are actu-
ally secreted by endothelial cells and correspondingly were
also found within the tissue interior to varying degrees.30 To
confirm the subendothelial localization of ADAMTS-13 and
VWF, immunostains were repeated on elderly, 2-year-old por-
cine aortic valve sample (OLD) tissues using new antibodies
specific for the proteins (Figure III in the online-only Data
Supplement). These additional immunostains showed the
same pattern.

Furthermore, previous studies have suggested that VEC dys-
function may play a role in initiating valvular diseases.24–27

Despite these links between VECs and valve diseases, little
has been accomplished to characterize the fundamental hemo-
static behavior of VECs and the relation between this behavior
and the valvular ECM in health, disease, and aging conditions.
It is well-known that vascular endothelial cells perform sub-
stantial antithrombotic roles via production and release of tis-
sue plasminogen activator (tPA), tissue factor pathway inhibitor

<table>
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<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>ADAMTS-13</td>
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<td>ADT</td>
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<td>AV</td>
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<td>CAVD</td>
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<td>ECM</td>
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<td>VWF</td>
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<td>YNG</td>
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However, the proportion of tissue stained for VWF was significantly higher in OLD tissues than in YNG tissues ($P<0.05$). There were trends of higher proportions of OLD valve tissues stained for PAI-1 ($P=0.06$) and TFPI ($P=0.07$) than in the corresponding portions of staining of YNG tissues, and a trend of higher proportion of TFPI staining in OLD tissues than in ADT tissues as well ($P=0.09$). The proportions of the valve tissues stained for PAI-1, TF, and tPA were low (<10%) among all age groups in comparison to the other proteins evaluated (≤40%; Figure 2), and these less abundant proteins were localized primarily in the endothelium (Figure 1).

**Porcine Aortic Valve Endothelial Cell Hemostatic Protein Gene Regulation Changes With Age**

Gene expression of the antithrombotic and thrombotic proteins was quantified with quantitative real-time polymerase chain reaction (Figure 3). Porcine aortic valve endothelial cell (PAVEC) gene expression in all age groups was quantified relative to porcine pulmonary artery endothelial cells (PPAECs; denoted by dotted lines in Figure 3). Gene expression for VWF in YNG PAVECs was significantly higher than in OLD PAVECs (48×; $P<0.005$). Similarly, gene expression

### Table. Antibodies Used for Immunohistochemistry and Immunocytochemistry

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Catalog No.</th>
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<tr>
<td><strong>Thrombotic proteins</strong></td>
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<tr>
<td>von Willebrand factor</td>
<td>Binds platelets to form platelet thrombi when in ultralarge multimer form</td>
<td>Ab6994*</td>
<td>1:200</td>
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<tr>
<td>Tissue factor</td>
<td>Surface protein expressed by activated endothelial cells to initiate coagulation cascade</td>
<td>SC18712†</td>
<td>1:50</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1</td>
<td>Tissue plasminogen activator inhibitor</td>
<td>SC6842†</td>
<td>1:100</td>
</tr>
<tr>
<td><strong>Antithrombotic proteins</strong></td>
<td></td>
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<td></td>
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<tr>
<td>A disintegrin and metalloproteinase with a thrombospondin type I motif, member 13</td>
<td>von Willebrand factor cleaving enzyme</td>
<td>BL-159‡</td>
<td>1:500</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor</td>
<td>Major inhibitor of tissue factor, factor Xa, and thrombin</td>
<td>SC18712†</td>
<td>1:100</td>
</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>Regulator of fibrinolysis</td>
<td>Bs-1545R§</td>
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<tr>
<td><strong>ECM proteins</strong></td>
<td></td>
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<tr>
<td>Collagen type I</td>
<td>Fibrillar collagen concentrated at fibroa of AV leaflet; most abundant</td>
<td>Ab34710*</td>
<td>1:50</td>
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<tr>
<td>Collagen type III</td>
<td>Fibrillar collagen found mainly in fibroa of AV leaflet</td>
<td>Ab7778*</td>
<td>1:500</td>
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<tr>
<td>Elastin</td>
<td>Highly elastic ECM component concentrated at ventricularr of AV leaflet</td>
<td>Ab9519*</td>
<td>1:50</td>
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AV indicates aortic valve; and ECM, extracellular matrix.

Corresponding manufacturers: *Abcam (Cambridge, MA); †Santa Cruz Biotechnology (Santa Cruz, CA); ‡Bethyl Laboratories (Montgomery, TX); §Bioss Laboratories (Woburn, MA).

**Figure 1.** Movat pentachrome (MOVAT) stains of aortic valve leaflet sections and higher magnification views of the midleaflet regions (indicated by the black box on MOVAT) for a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13 (ADAMTS-13), tissue factor pathway inhibitor (TFPI), tissue plasminogen activator (tPA), von Willebrand factor (VWF), tissue factor (TF), and plasminogen activator inhibitor 1 (PAI-1). ADT indicates 6-month-old porcine aortic valve sample; f, fibrosa; OLD, 2-year-old porcine aortic valve sample; s, spongiosa; v, ventricularis; and YNG, 6-week-old porcine aortic valve sample.

**Figure 2.** Semiquantitative analysis results of the proportion of assessed tissue stained for the hemostatic proteins in the differently aged aortic valve leaflets. *$P<0.05$ between noted age groups for that protein. ADAMTS-13 indicates a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13; ADT, 6-month-old porcine aortic valve sample; OLD, 2-year-old porcine aortic valve sample; PAI-1, plasminogen activator inhibitor 1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; tPA, tissue plasminogen activator; VWF, von Willebrand factor; and YNG, 6-week-old porcine aortic valve sample.
for the antithrombotic proteins TFPI and tPA was 3-fold higher in YNG PAVECs than in OLD PAVECs ($P<0.05$ for each). Compared with the PPAEC control group, the YNG PAVEC gene expression was significantly higher for VWF (58×; $P<0.0005$), TFPI (3×; $P<0.05$), and tPA (30×; $P<0.05$).

There were no significant differences between ADT PAVECs and the other 2 age groups for all proteins. However, ADT PAVECs did have significantly higher gene expression of TFPI (2.5×; $P<0.05$) compared with the PPAEC controls.

The only protein that had the highest expression in the OLD PAVECs was the thrombotic protein PAI-1, which was 2-fold higher than in both YNG and ADT PAVECs ($P<0.05$). There were no significant differences in the gene expression for ADAMTS-13 and TF between the PAVEC groups and PPAEC controls. Furthermore, all PAVEC and PPAEC groups had $\approx 1000\times$ higher gene expression levels for all hemostatic proteins than did human umbilical vein endothelial cells (HUVECs; data not shown).

PAVECs Synthesize Hemostatic Mediators In Vitro

Primary PAVECs harvested from differently aged AV tissues maintained their endothelial phenotype and stained positively for CD31 (data not shown). ADAMTS-13 and VWF were generally colocalized in all age groups, although there was a greater abundance of VWF staining overall (Figure 4, first column). However, VWF levels in the OLD PAVECs appeared more profuse than in the YNG PAVEC cultures.

PAVECs positively stained for low levels of TFPI and TF (Figure 4, second column). TFPI staining in YNG and ADT PAVECs was primarily localized intracellularly with some punctate staining outside of the cells; TFPI staining in OLD PAVECs was low, with punctate staining outside of the cells. For the YNG PAVEC cultures, there seemed to be higher levels of TFPI than of TF. Conversely, TF was more abundant than TFPI in OLD cultures.

PAI-1 and tPA were also detected in PAVEC cultures (Figure 4, third column). There was intracellular staining for PAI-1 in all PAVEC age groups, although punctate extracellular staining of PAI-1 was present in the OLD PAVEC cultures. Staining for tPA appeared to be more abundant in YNG and ADT PAVEC cultures than in OLD PAVEC cultures. Although the stains for PAI-1 and tPA were generally less abundant in the OLD PAVEC cultures, the complimentary proteins seemed to be colocalized with slightly higher levels of PAI-1.

PAVECs Can Mediate Functional VWF Release and Cleavage

Histamine stimulation of PAVECs in vitro initiated the rapid release of thrombogenic VWF multimer proteins into the culture medium. For the comparison of PAVEC age groups, VWF protein release paralleled the VWF gene expression trend in that YNG PAVECs released a significantly higher concentration of VWF protein than did OLD PAVECs ($P<0.05$; Figure 5A). VWF release by the PPAECs and ADT PAVECs was not significantly different from that released by the YNG and OLD PAVECs. HUVECs released significantly higher amounts of VWF protein than all porcine-derived PAVEC and PPAEC groups ($P<0.001$).

The fraction of cleaved VWF-140 fragments was measured using enzyme-linked immunosorbent assays with a specific

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**Figure 3.** Porcine aortic valve endothelial cell (PAVEC) gene expression of antithrombotic (A–C) and thrombotic (D–F) proteins relative to porcine pulmonary artery endothelial cells (PPAECs). $\Delta P<0.05$ between noted PAVEC age group vs the relative PPAEC gene expression (indicated by dashed line). $\Delta P<0.05$ between noted aged age groups for that protein. ADAMTS-13 indicates a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13; ADT, 6-month-old porcine aortic valve sample; OLD, 2-year-old porcine aortic valve sample; PAI-1, plasminogen activator inhibitor 1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; tPA, tissue plasminogen activator; VWF, von Willebrand factor; and YNG, 6-week-old porcine aortic valve sample.

**Figure 4.** Representative immunofluorescent stains of antithrombotic (a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13 [ADAMTS-13], tissue factor pathway inhibitor [TFPI], tissue plasminogen activator [tPA]) proteins (green) and thrombotic (von Willebrand factor [VWF], tissue factor [TF], plasminogen activator inhibitor [PAI-1]) proteins in 6-week-old porcine aortic valve sample (YNG) and 2-year-old porcine aortic valve sample (OLD) PAVECs. Cell nuclei were stained with DAPI (scale bar=100 µm).
monoclonal antibody for cleaved VWF proteins. There was no statistically significant difference in the fraction of VWF-140 fragments between porcine-derived PAVECs and PPAECs (Figure 5B). For the HUVECs, only ≈10% of the VWF protein was cleaved into VWF-140 fragments, which was a significantly lower cleavage product fraction compared with the OLD PAVECs (P<0.05).

**PAVEC-Released VWF Increases Porcine Aortic Valve Interstitial Cell Calcific Nodule Formation In Vitro**

Porcine aortic valve interstitial cells (PAVICs) were cultured with various culture medium conditions to assess how the addition of VEC-released VWF affect calcific nodule formation in vitro. Measured levels of VWF protein present in the low serum PAVIC medium mixed with 3% (v/v) conditioned culture mediums collected from YNG, ADT, and OLD PAVEC groups were 257, 130, and 90 pg/mL, respectively. No levels of VWF protein were detected in any of the 3 control conditions. Within 5 days of culture, nodule formation was visible in PAVIC cultures treated with each of the conditioned mediums from the differently aged PAVECs. At the end of the 10-day culture period, there were no significant differences in average number of nodules per well, average nodule size, or total calcified area between the control groups of low serum PAVIC medium, 3% PAVEC stimulation medium without histamine, and 3% PAVEC stimulation medium with histamine (Figure 6 and Figure VI in the online-only Data Supplement). The total calcified area in PAVICs treated with the conditioned mediums from YNG, ADT, and OLD PAVEC supernatants (2.0–2.4×10^4 µm^2) were all significantly greater than all 3 control groups (1.2–3.2×10^4 µm^2; P<0.001; Figure 6). PAVICs in both YNG (40 nodules) and ADT (45 nodules) conditioned medium groups had significantly more nodules per well than the 3 control groups (9–13 nodules; P<0.001; Figure VIA in the online-only Data Supplement). Furthermore, the average nodule size in PAVICs treated with 1 of the 3 conditioned mediums (52–78×10^3 µm^2) were significantly larger than each of the 3 control conditions (13–23×10^3 µm^2; P<0.001; Figure VIB in the online-only Data Supplement).

**Discussion**

The endothelial cell–mediated process of hemostasis is essential for the function of all living heart valve tissues. As these tissues undergo remodeling with age and disease, VEC management of hemostatic protein regulation also changes. This study is the first to examine the production and expression of numerous hemostatic proteins in AV tissues and in vitro PAVEC cultures from 3 distinct age groups. There are several age-related differences in the abundance and localization of thrombotic and antithrombotic proteins within the AV. As expected, all examined hemostatic proteins were strongly present at the leaflet edges in each age group. However, many of these soluble components, namely ADAMTS-13, TFPI, VWF, TF, and PAI-1, were found throughout the interior of the valves as well. These proteins were primarily localized in the ventricularis layer of the AV in YNG and ADT tissues. Conversely, they were distributed more evenly across the valve layers in OLD AV samples. Because of their interior location, it is possible that these hemostatic components interact with subendothelial ECM components such as elastin and collagen type I. The ventricularis layer contains densely packed elastin, which may sequester the soluble hemostatic proteins that have permeated into the subendothelium. As elastin becomes more disperse throughout the OLD AV tissues,10 the hemostatic proteins may be able to permeate throughout the entire interior of the AV.

These proteins may also interact with collagen type I because it is the most abundant valve ECM protein and is present throughout the leaflet. Previous work investigating fiber alignment in articular cartilage tissues suggests that highly organized collagen alignment limits diffusion of proteins through anisotropic tissues.31,32 Similarly, the lower amount of hemostatic proteins within the fibrosa layer of the YNG and ADT AV tissues may be attributable to the highly organized and aligned collagen fibrils serving as a barrier to limit the diffusion of hemostatic proteins into the aortic side of the AV leaflet. Given the age-associated collagen remodeling and turnover observed in older tissues,14 this barrier function of the fibrosa may be attenuated with age and may result in the increased permeation of VEC-secreted hemostatic proteins throughout the valve layers.

In addition, quantitative real-time polymerase chain reaction analyses showed highly significant changes in gene expression of many hemostatic proteins with age in PAVEC cultures. PAI-1, the protein inhibitor for tPA in the fibrinolysis pathways, was significantly elevated with age, as shown by mRNA
analysis, immunocytochemistry, and a trend in the proportion of tissue stained. The increased accumulation of PAI-1 in older valves suggests that this smaller-molecular-weight protein can easily permeate through the remodeled ECM architectures in OLD valves. Previous studies have shown that inflammatory or cytokine stimuli can increase endothelial secretion of PAI-1 while not affecting tPA secretion.\textsuperscript{30,33} It seems likely that elderly patients with higher levels of inflammation, cholesterol, and tissue remodeling will also experience more procoagulant states in which PAI-1 levels in the blood and ECM overcome the basally secreted levels of tPA.\textsuperscript{33–35} Interestingly, there were no differences in tPA accumulation in AV tissues despite the significant decline in tPA gene expression in OLD PA VECs relative to YNG PA VECs. This may suggest that the expressed tPA is released more into the bloodstream as opposed to accumulating within valve tissues. The reduction in PA VEC tPA expression also reflects the hemostatic imbalance between tPA and PAI-1 associated with age.

PAVEC expression and tissue accumulation of TFPI and VWF were affected by age-related changes as well. In both cases, YNG PAVECs had significantly higher levels of TFPI and VWF gene expression than did OLD PAVECs. Consistent with quantitative real-time polymerase chain reaction results, stains for TFPI in YNG PAVECs appeared to be more abundant than in OLD PAVEC cultures. Conversely, OLD PAVEC cultures had slightly higher levels of VWF staining than in YNG PAVEC cultures. However, the polyclonal anti-VWF antibody used in the stain binds to both cleaved and uncleaved VWF proteins. Therefore, the increased staining observed in the OLD PAVEC cultures is likely from the higher proportion of cleaved VWF proteins in the condition, as observed in the enzyme-linked immunosorbent assay experiments. Furthermore, YNG valves showed trends of lower proportions of tissue stained for both TFPI and VWF than in OLD tissues. This discrepancy could be a result of the retention of these basally secreted proteins within the disorganized OLD tissue ECM through interactions with heparin and collagen type I.\textsuperscript{30,36}

PAVEC expression of ADAMTS-13 and TF was not affected by age for gene expression or the proportion of tissue stained. Thus, these specific hemostatic proteins seem less likely to be direct players in causing age-related valve disease. These results, however, suggest that the valvular hemostasis may become unbalanced over a lifetime because many of the associated counteracting hemostatic proteins (VWF for ADAMTS-13, TFPI for TF) were shown to be affected by age. This imbalance was also observed in the immunostaining of PAVECs because colocalization of the associated hemostatic component pairs was less present in the OLD PAVEC cultures.

Functional testing of the fragmentation of histamine-stimulated VWF confirmed that PAVECs secrete ADAMTS-13 to cleave VWF multimers. The total amount of VWF protein released by OLD PAVECs was significantly lower than that released by YNG PAVECs, which was consistent with the gene expression results. The fractions of cleaved VWF (140 kDa fragments) were not significantly different between all porcine-derived endothelial cell groups, which supports the previous finding that ADAMTS-13 expression and function is not affected by age.\textsuperscript{37} On the contrary, because VWF expression and release are affected by age, and with the elderly age group having a decline in VWF expression and production, there will be lower proportions of complete VWF proteins present in patient plasma that can mediate coagulation. This phenomenon is consistent with clinical reports that elderly patients require lower doses of anticoagulants after heart valve surgeries than do adult patients because of lower clearance kinetics of the drugs and decreased clotting ability in elderly patients.\textsuperscript{38}
The changes in hemostatic protein regulation by VECs from our results match the age-related hemostatic protein plasma levels previously reported.\textsuperscript{33–35,38} We think that these age-related imbalances in hemostatic protein regulation and production by VECs are not likely to cause spontaneous development of thrombus. However, when injury or insult occur at the valve endothelium, the aging-induced imbalance in the expression of antithrombotic and thrombotic proteins by VECs will lower the capability of the endothelium to maintain hemostasis and thus cause AV tissues to be more susceptible to thrombotic complications. Also, injury to the endothelium exposes the underlying tissue, and therefore the accumulation of these proteins in valve tissues along with the structural changes can induce further thrombotic events.

Aside from intravascular complications, accumulation of these proteins in valve tissues along with the structural changes can cause tissue-level progression of age-related diseases such as calcific aortic valve disease (CAVD). Using an in vitro CAVD model, PAVIC nodule formation experiments showed that the presence of VEC-released VWF significantly increased the total number of nodules, nodule area, and total calcified area in PAVICs relative to control groups. Having no differences in nodule formation between the 3 control culture conditions confirmed that the significant increase in calcific nodule formation by PAVICs was not because of the addition of PAVEC stimulation medium or residual levels of histamine in solution, but rather because of the presence of VEC-released proteins including VWF after histamine stimulation. Therefore, the secretion and accumulation of VWF and other hemostatic proteins within the valve tissues may affect the development and progression of CAVD and other acquired valvular diseases.

Although there were no significant differences in PAVIC nodule formation between the differently aged PAVEC-based conditions, the significant increases in VWF present within OLD valve tissues in conjunction with age-associated ECM disorganization seen in the histology slides suggest that calcification could result from increased pro-osteogenic VWF–valvular interstitial cell interactions, which correlated with the classical clinical presentation of CAVD in the elderly. Although VWF is the main component of histamine-stimulated PAVEC supernatant, other factors in the supernatant (e.g., prostacyclin, platelet-activating factor, angiotryptin-2, and interleukin-8) may also intensify PAVIC nodule formation, and further investigation into how other hemostatic proteins within valve tissues can affect AV calcification is warranted.\textsuperscript{39–41} Nonetheless, our studies have shown that PAVECs will constitutively secrete VWF and other hemostatic factors, which will result in their accumulation of these proteins within the valve subendothelium. As ECM organization changes with age and the valve hemostatic protein regulation becomes more imbalanced, there is the potential that VWF and some of these proteins may play a substantial role in the progression of age-related valve disease.

This study was also the first to perform an extensive characterization of PAVEC hemostatic protein expression and production relative to vascular endothelial cell types PPAECs and HUVECs. PPAECs have been documented to produce significant levels of VWF and were chosen as a porcine vascular endothelial cell control group.\textsuperscript{42} Previous findings have found that porcine endothelial cultures isolated from the thoracic aorta expressed low levels of VWF mRNA and did not contain Weibel–Palade bodies, indicating that porcine endothelial cells have different regional capacities for hemostatic regulation.\textsuperscript{52–53} The gene expression of TFPI in YNG and ADT PAVECs and of tPA and VWF in YNG PAVECs were significantly higher than in the baseline PPAECs, suggesting that PAVECs have distinct hemostatic regulation activity. Gene expression levels differed greatly between PAVECs and HUVECs, with expression of the investigated proteins being \textsuperscript{3} orders of magnitude higher in the PAVECs. HUVECs have been well-documented to produce and release VWF in vitro\textsuperscript{50,54} and thus are frequently used in endothelial cell studies and hemostatic experiments. However, the concentration of VWF protein released from histamine-stimulated HUVECs was significantly higher than all porcine-derived endothelial cell groups, suggesting that HUVECs are capable of storing more VWF in their Weibel–Palade bodies compared with porcine endothelial cells. Therefore, future studies regarding PAVEC expression and mediated protein functions should consider using porcine-derived vascular endothelial cells as baseline controls.

Limitations of this study include the inherent variability in the semiquantitative analysis of immunohistochemistry, which reduces the ability to distinguish between age groups. A larger sample size may help in confirming the promising trends. Still, this study examined several key endothelial cell–mediated hemostatic proteins. Additional factors such as ectonucleotidases, prostacyclin, and thrombomodulin are yet to be studied with respect to PAVEC hemostatic regulation. Future studies using a gene array for a wider panel of thrombotic and antithrombotic proteins in conjunction with ECM components and inflammatory markers may be informative in elucidating the relationship between the VECs regulation of their environment and how the protein accumulation influences the biology and pathology of the AV. Furthermore, although the interaction and balance of thrombotic and antithrombotic proteins are well understood at the apical side of the endothelium, further studies to investigate the interactions of each protein with subendothelial ECM and cells may be important in understanding the mechanisms that can promote the onset of valvular dysfunction. Finally, further investigation into the molecular mechanisms behind the age-related imbalances in PAVEC hemostatic protein regulation will greatly enhance our knowledge of these changes and may provide potential targets for the prevention and treatment of acquired valve abnormalities.

In conclusion, it is well-understood that valve tissues experience different environments with respect to age as a result of changes in valve mechanics, hemodynamics, and matrix composition. This study has identified age-related differences in PAVEC hemostatic protein regulation and the ability of basally secreted proteins to aggregate within differently aged AV tissues. In addition, we have verified that PAVECs express many of the same thrombotic and antithrombotic proteins as vascular endothelial cells. Although there was not one pattern for all hemostatic protein expression and aggregation with respect to age, we demonstrated age-related differences in the overall expression and localization of tPA, PAI-1, TFPI, and VWF in AV tissues and cells. Although thrombosis does not commonly
occur as an age-associated dysfunction in valve tissues, we observed that thickened elderly valve tissues with ubiquitous distributions of elastin and collagen sequester high levels of the hemostatic proteins in the subendothelium, which warrants further investigation into potential roles for these aggregated proteins in the formation of calcific nodules that are so prevalent in older AVs. Our in vitro PAVIC studies suggest that the presence of these VWF promotes PAVIC calcification and that VWF and other hemostatic proteins within the tissue interior may have a potential role in the development of CAVD and other acquired abnormalities as age-related valve tissue ECM remodeling occurs. Thus, future studies regarding valve biology, pathology, and tissue engineering require consideration of environments that reflect the specific age group in question, including the necessary hemostatic framework.

Acknowledgments

We thank Dr Joel L. Moake for extensive project support including discussion of the data and manuscript. We thankfully acknowledge Nancy Turner and Leticia Nolasco for providing harvested primary HUVECs and guidance in conducting VWF release and cleavage assays. We also thank Adam Lin for his thoughtful discussion of the study results.

Sources of Funding

This research was funded by a predoctoral fellowship from the American Heart Association (to L.R. Balaoing), the National Institutes of Health (HL110063), the Mary R. Gibson Foundation, and the Mabel and Everett Hinkson Memorial Fund.

Disclosures

None.

References

Valvular endothelial cells (VECs) play an important role in maintaining valve homeostasis and affect the onset of valve disease. However, little has been done to characterize the fundamental hemostatic behavior of VECs and its relation to the extracellular matrix in health, disease, and aging. This study was the first to perform an extensive age-dependent characterization for hemostatic proteins in valves and VEC hemostatic regulation relative to vascular endothelial cell types. Results identified age-related differences in VEC hemostatic protein regulation, and the increased capacity of specific proteins to aggregate within regions of elderly valves have been shown to have age-associated loss of extracellular matrix organization. In vitro calcific aortic valve disease model studies show that the presence of von Willebrand factor significantly increases valvular interstitial cell formation of calcific nodules relative to baseline controls. Therefore, the hemostasis imbalance with aging and the accumulations of hemostatic proteins may contribute to the formation of calcific nodules and warrants further investigation to determine the connection between VEC hemostatic mechanisms and the progression of valve disease. Furthermore, this works proposes the importance of selecting appropriate age- and species-based controls in future VEC and hemostatic work.

Significance
Materials and Methods

Preparation of sample groups

Aortic valve leaflets were dissected from fresh porcine hearts acquired from commercial abattoirs (Fisher Ham and Meats, Spring, TX; Animal Technologies, Tyler, TX), and assigned into one of three age groups: young (YNG=6 week old), adult (ADT=6 month old), or older (OLD=2 years old). Aortic valve leaflet tissues were either dehydrated, processed in paraffin and radially sectioned for \textit{in situ} analysis, or enzymatically digested in a solution of DMEM containing dispase (2 U/mL) and collagenase II (60 U/mL) to isolate porcine aortic valvular endothelial cells (PAVECs) from the valve surfaces for cell culture following previously described methods.\textsuperscript{1,2} At first passage, the VECs were purified using CD31 antibody conjugated-\textsc{C}ELLection magnetic sorting beads (Invitrogen, Carlsbad, CA). Porcine pulmonary artery endothelial cells (PPAECs) and human umbilical vein endothelial cells (HUVECs) were used as baseline controls for \textit{in vitro} experiments. PPAECs were isolated from the lumen of fresh porcine pulmonary artery tissue following the same procedures described for the PAVEC isolations. HUVECs were isolated from umbilical cord tissues acquired from St. Luke’s Episcopal Hospital, Houston, TX, following previously described methods.\textsuperscript{3} All cells were cultured on tissue culture plastic previously coated with a 2.5% gelatin solution and supplemented with specialized EGM-2 medium (Lonza, Walkersville, MD) with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin in an incubator (37°C, 5% CO\textsubscript{2}, 95% humidity). Cell culture medium was changed every 2-3 days, with cell passaging when confluence reached 85%.

Investigated hemostatic mediators

Known vascular EC-expressed thrombotic proteins were used to assess the hemostatic capacity of VECs from different aged specimens. Antibodies against thrombotic proteins (von Willebrand Factor [VWF] (Abcam, Cambridge, MA), tissue factor [TF], and plasminogen activator inhibitor-1 [PAI-1]) as well as anti-thrombotic proteins (VWF cleaving enzyme [ADAMTS-13] (Bethyl Laboratories, Montgomery, TX), tissue plasminogen activator [tPA] (Bioss Laboratories, Woburn, MA), and tissue factor pathways inhibitor [TFPI]) were used for immunohistochemistry and immunofluorescence to assess mediator localization and production. All antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), unless otherwise noted. (See Table 1 for summary of antibodies).

Histology and Immunohistochemistry

Paraffin embedded and sectioned valve tissue samples were stained with Movat’s pentachrome (MOVAT) to identify differences in ECM organization and composition between the three age groups. The MOVAT used a series of tissue processing and chemical stains to dye cell nuclei purple, collagen yellow, proteoglycans and glycosaminoglycans blue, elastic fibers black, fibrin dark red, and muscle red. To localize the endothelial cell produced-hemostatic mediators in valve tissues, immunohistochemistry (IHC) was performed using the primary antibodies listed in Table 1 and biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA) and visualized using a 3,3’-Diaminobenzidine (DAB) chromagen reaction (Vector Laboratories, Burlingame, CA) with a hematoxylin-2 counterstain for cell nuclei. All immunostained tissue specimens were pretreated with Citrate Buffer Antigen Decloaker (Biocare Medical, Concord, CA) for 30 min at 80°C, and blocked with 1% donkey serum buffer (DSB) (GeneTex, Irvine, CA) for 1 hr at room temperature. A negative control for each stained section remained incubated in DSB, while primary antibodies were incubated overnight at 4°C. Biotinylated secondary antibodies were incubated on all
samples, including negative controls, for 1 hr at room temperature prior to Vectastain and DAB treatments. Stained sections were compared with parallel tissue sections immunostained for the ECM components collagen type I, collagen type III, and elastin (Abcam). Whole tissue images, as well as magnified images of the midleaflet region of each tissue specimen, were generated using a PathScan Enabler slide scanner (Meyer Instruments, Houston, TX) and Leica DMLS upright light microscope (Buffalo Grove, IL), respectively.

Semi-quantitative analysis of the DAB chromagen levels was performed at the midleaflet region of each tissue (n=3-6 valves per age group). All histological and immunohistochemical images that were analyzed and compared with one another were captured on the same microscope, at the same magnifications and exposure, with white balancing performed on only the first sample of the set. Grading of DAB staining intensity was performed using ImageJ Software (NIH, Bethesda, MD). A background subtraction of 150 pixel rolling ball radius was applied to an image, then the image was processed using a Color Deconvolution plugin developed by A.C. Ruifrok to separate hematoxylin channels (nuclear counterstain) from the antigen-positive DAB stain channels. A binary mask was created by taking the threshold of the DAB channel images at the minimum threshold intensity measured in the negative control tissue samples. The DAB intensity and associated areas of the antigen-positive regions were quantified by applying the binary mask to the original sample image and analyzing particles such that the positive areas stained were recorded in pixels. The output data was used to quantify the proportion of tissue area stained as: \( \frac{\sum a_i}{A} \), where \( a_i \) was the measured section of stained area, and \( A \) was the total sample area in the image field of view.

**Immunocytochemistry**

Hemostatic mediator antibodies (at the same dilution used in IHC) were used with fluorescent Alexa-fluor secondary antibodies (Invitrogen) to localize and verify PAVEC production of the above noted hemostatic proteins *in vitro*. PAVECs were fixed with 2% paraformaldehyde for 10 min after 5-7 days in culture on gelatin coated chamber slides. The fixed cells were permeabilized with 0.2% Triton-X for 10 min, then blocked with DSB for 1 hr prior to the addition of primary and secondary antibodies. Cells were also immunostained for the endothelial marker CD31 (Abcam) to ensure that the PAVECs had not undergone any change in cell phenotype. Fluorescence imaging was performed using a Zeiss LSM 5Live Confocal Microscope (Zeiss, Oberkochen, Germany).

**Quantitative RT-PCR**

RNA from the cell cultures was extracted using Trizol Reagent (Invitrogen) and a series of ethanol centrifugations. The mRNA was reverse transcribed into cDNA using Primerscript 1st Strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). Quantitative RT-PCR (qRT-PCR) was performed on the cDNA using 2X QuantiTect Sybr Green PCR Master Mix (Clontech, Mountainview, CA) with a Mastercycle ep Realplex (Eppendorf, Hamburg, Germany) to measure differences in gene expression levels for hemostatic mediator proteins between the YNG, ADT, and OLD PAVECs, PPAECs, and HUVECs (n=5-6 samples per cell type). QRT-PCR was performed in each sample in triplicates. The GAPDH gene was used as a housekeeping gene, and sample group protein gene expression was normalized to the corresponding PPAECs gene expression levels following the mathematical model for relative qRT-PCR. All DNA primers were
purchased from Integrated DNA Technologies (Coralville, IA; see Table SI for DNA primer sequences).

**VWF release and cleavage assay**

Previous work has shown that the addition of histamine to HUVEC cultures *in vitro* effectively initiates endothelial cell secretion of hyper-thrombotic ultra-large VWF multimer chains previously stored in Weibel-Palade bodies at the cell membrane, while leaving EC expression and release levels of the VWF cleaving enzyme ADAMTS-13 unchanged. The quantities of total VWF protein or inactivated, cleaved VWF fragments in the solution were measured using enzyme linked immunosorbent assays (ELISAs) to assess the functionality and capacity of ADAMTS-13 enzyme to cleave VWF.

PAVECs, PPAECs, and HUVECs (n=6) were incubated with PAVEC stimulation medium consisting of serum-free EGM-2 media (with 1% v/v of insulin-transferring selenium A (Sigma-Aldrich, St. Louis, MO) and 1% (w/v) BSA (Sigma-Aldrich)) containing 100 μM of histamine at 37°C for 10 min. Next, the culture medium was collected with 10 mM EDTA and stored at -20°C until use. Maxisorb 96-well plates (Thermo Scientific, Waltham, MA) were coated with 1 μg/mL of polyclonal rabbit anti-porcine VWF antibody (Abcam) in a Coating Solution buffer (KPL, Gaithersburg, MD) overnight at 4°C. The wells were then blocked with 1% w/v BSA/PBS solution for 1hr at 37°C. Samples of cell media supernatant or pooled human plasma were diluted with 1% BSA/PBS solution and incubated in the wells for 1 hr at 37°C. After washing, wells were incubated with 1 μg/mL of detection antibody for mouse anti-porcine full length VWF protein monoclonal antibody (2Q2134, Abcam) or mouse anti-human VWF 140-kDa fragment antibody (amino acids L1591-Y1605, Bethyl Laboratories) for 1 hr at 37°C. The mouse detection antibodies were tagged with 1 μg/mL of peroxidase-labeled anti-mouse IgG (KPL) and then incubated with SureBlue Reserve TMB peroxidase solution (KPL) to expose the peroxidases on the bound detection antibodies. The reaction was stopped with the addition of TMB Stop Solution (KPL), and the 450 nm absorbance of each well was read using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA). Serial dilutions of human plasma were used to create a standard curve, with the assumption of 10 μg/mL of VWF present per mL of pooled plasma.

**Calcific nodule formation assay**

To assess whether VEC-released VWF has a role in the development of CAVD, a CAVD *in vitro* model in which primary porcine aortic valvular interstitial cells (PAVICs) were cultured with various conditioned culture mediums to observe calcific nodule formation was performed. Following the VWF release protocol used above, 5 mL of PAVEC stimulation medium supernatant (from histamine stimulated PAVECs in T-75 culture flasks) were collected after 10 min of incubation at 37°C. The collected PAVEC treated medium for each age group was mixed at 3% (v/v) with low serum PAVIC culture medium (48% DMEM, 49% F12, 1% HEPES, 1% ABAM, 1% BGS), aliquoted and frozen at -20°C until use.

Concentrations of VWF protein from each conditioned medium group were quantified using VWF sandwich ELISA. Histamine is an inflammatory mediator that is found in almost all tissues and can affect PAVIC proliferation and nodule formation *in vitro* at high concentrations (unpublished observations). Therefore, to provide an appropriate control, the levels of residual histamine within the conditioned PAVEC culture mediums were measured using a histamine ELISA (Genway Biotech, San Diego,
The average residual histamine levels from the different aged PAVEC conditioned mediums was found to be 8.4nM.

Three control groups were prepared. First, low serum PAVIC culture medium served as a baseline condition for calcific nodule formation based on previous work (cite). Second, low serum PAVIC culture medium mixed with 3% (v/v) fresh PAVEC stimulation medium without histamine served as an additional control group. Third, to ensure the addition of residual histamine was not the cause of PAVIC nodule formation, another group of PAVICs were treated with low serum PAVIC culture medium mixed with 3% (v/v) fresh PAVEC stimulation medium with 8.4 nM of histamine was used.

PAVICs here harvested from porcine aortic valve leaflets and cultured in PAVIC growth medium (10% BGS) following previously described methods. At P2-P3 PAVICs were seeded into 24 well plates at 50,000 cells/cm². PAVICs groups were cultured at 37°C, 5% CO₂. The culture mediums were replenished every other day. At day 10, the cultured PAVICs were fixed with 4% paraformaldehyde, and stained with 40mM Alizarin Red S (ARS) for 30 min. After the dye was removed and rinsed, it could be observed that the calcific nodules were stained red. A photomask consisting of a circle divided into four quadrants was applied to each well of the culture plate in a way that excluded the outer 2.25mm edges of the well. Each well was imaged in quadrants using a Zeiss Stemi 200C stereoscope with a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, MI). Using image J software threshold and particle count functions, the total nodule count and respective area was quantified for two quadrants of each well and averaged per culture condition to quantify average total calcified area per well.

**Statistical Analysis**

One way analysis of variance (ANOVA) statistics and Tukey post hoc tests were performed to compare quantified values between different aged tissue and PAVEC groups, and PPAEC and HUVEC control groups. ANOVA and Tukey post hoc tests were also performed to compare calcified values between PAVIC groups treated with different aged-conditioned mediums and the control culture medium groups. P-values <0.05 were considered as significant for all studies.
References


Table SI. Summary of hemostatic protein DNA primer sequences used for qRT-PCR.

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Figure S1. MOVATs of stained sections of aortic valve leaflets and higher magnification views of immunostained midleaflet regions (indicated by red box on MOVAT) for extracellular matrix components COL I, COL III and Elastin. (f: fibrosa, s: spongiosa v: ventricularis).
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**Figure SII.** Immunohistological stains and the associated negative controls for ADAMTS-13, TFPI, tPA, VWF, TF, and PAI-1 associated with Figure 1.
**Figure SIII.** Immunostains comparing endothelium and subendothelial localizations for (A) ADAMTS-13 and (B) VWF in OLD AV tissues between original antibodies (bold) and additional antibodies. (C) Summary of ADAMTS-13 and VWF antibodies used for immunostains. Corresponding manufacturers: a. Bethyl Laboratories (Montgomery, TX) b. Abcam (Cambridge, MA).
Figure SIV. Immunofluorescent stains of anti-thrombotic (ADAMTS-13, TFPI, tPA) proteins (green), and thrombotic (VWF, TF, PAI-1) proteins (red) in YNG PAVECs corresponding to merged stains in Figure 4. Cell nuclei were stained with DAPI (blue). Scale bar = 100µm.
Figure SV. Immunofluorescent stains of anti-thrombotic (ADAMTS-13, TFPI, tPA) proteins (green), and thrombotic (VWF, TF, PAI-1) proteins (red) in OLD PAVECs corresponding to merged stains in Figure 4. Cell nuclei were stained with DAPI (blue). Scale bar = 100µm.
Figure SVI. Quantification of the average PAVIC nodules per well (A) and average PAVIC calcified nodule size (B) for the different aged PAVEC-released VWF conditioned mediums and the three control groups. * = p-value <0.001, ^ = p-value<0.01.