Smooth Muscle Cell Proliferation in Response to Endothelial Injury in Coronary Arteries of Normal and von Willebrand’s Disease Swine

Mary Ann Lamb, James E. Manning, Robert L. Reddick, and Thomas R. Griggs

The proliferative response of medial smooth muscle cells to balloon-induced endothelial denudation was measured in coronary arteries of normal and von Willebrand’s disease swine, a strain of swine with a genetic defect affecting platelet-vessel wall interactions. Animals were sacrificed 48 hours following endothelial injury. Incorporation of a 1-hour pulse label of 3H-thymidine was determined by measuring DNA specific activity. Autoradiography of coronary artery step segments was also performed. The ratio of DNA specific activity in ballooned vessels to that in nonballooned vessels ranged from 1.2 to 26.2. No statistically significant difference in 3H-thymidine incorporation between the two phenotypes was documented (p > 0.25). Similarly, DNA incorporation of 3H-thymidine as determined by autoradiography was accelerated to the same degree in ballooned vessels in both phenotypes. The results suggest that von Willebrand factor-mediated platelet activation is not essential for the release of platelet-derived growth factor or that in vivo factors other than platelet-derived growth factor play a significant role in the response of medial smooth muscle cells to acute intimal injury causing endothelial denudation.

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bleeder pigs. Studies of the acute effects of the balloon-catheter injury have shown that the density of adherent platelets on the denuded coronary subendothelium is similar in the two phenotypes. However, the platelets on denuded surfaces in the bleeder pigs have fewer pseudopodia and appear more rounded than do the platelets in the normal animals; this is evidence of a less active state. To further investigate the relationship between platelet adherence and activation and smooth muscle cell proliferation, we determined the degree of SMC growth in both normal and vWD swine by measuring the incorporation of $^3$H-thymidine into the DNA of SMC following injury to coronary artery endothelium. Despite evidence of impaired platelet activation in vWD have a prolonged skin bleeding time, reduced levels of FVIII coagulant activity in their plasma, and undetectable levels of platelet-aggregating factor/von Willebrand factor (PAF/vWF). Experiments were performed on 12 normal and 10 vWD swine.

Swine

Our animal care facility is accredited by the American Association for Accreditation of Animal Laboratory Care, and all procedures were reviewed and approved by the veterinary staff of the Division of Laboratory Animal Medicine. The animals used in this study were from the inbred strain of swine with vWD from the Chapel Hill colony. Homozygotes for vWD were used to evaluate SMC response to acute injury. All other experiments were performed on pairs of pigs, one normal and one vWD. The hemostatic profile of the experimental pigs is shown in Table 1.

### Methods

#### Chemicals

Chemicals were reagent grade or better unless otherwise indicated. All solutions were prepared with glass-distilled, deionized water.

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#### Table 1. Hemostatic Profile of Experimental Pigs

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean PAF/vWF (% normal)</th>
<th>Mean bleeding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>121 ± 45</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>vWD</td>
<td>&lt;1%</td>
<td>&gt;15</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>


#### Endothelial Denudation

Endothelial denudation was performed as previously described with modifications. Animals were anesthetized with halothane. Surgery was performed under sterile conditions. A Swan Ganz 4F catheter (Edwards Laboratories, Santa Ana, California) was passed through the surgically exposed carotid artery and directed into one of the three major coronary arteries, usually the left anterior descending (LAD). The other two arteries, the circumflex (CIRC) and the right coronary artery (RCA), were used as noninjured control vessels. The positioning of the catheter was monitored by fluoroscopy. The balloon at the tip of the catheter was inflated with 0.3 ml air. The inflated balloon was immediately withdrawn, limiting the interruption of blood flow to the injured coronary artery to less than 2 seconds. The inflated balloon was usually passed through the artery three times. The same operator performed the endothelial denudation procedure on all experimental pigs. No bleeding complications occurred in the bleeder animals, and no plasma replacement therapy was used. Figure 1 illustrates the degree and extent of injury typically obtained with this procedure.

#### Pulse Labeling with $^3$H-Thymidine

Forty-eight hours after balloon injury, each pig was given an intravenous injection of 0.5 mCi $^3$H-thymidine (sterile aqueous, specific activity > 10,000 mCi/mM, Schwartz-Mann, Incorporated, Orangeburg, New York) per kg body weight. Following a 1-hour pulse label, the animals were sacrificed with a lethal dose of anesthetic, and the heart was immediately removed.

#### Determination of Smooth Muscle Cell Proliferation

Smooth muscle cell proliferation was assessed by two methods, DNA synthetic activity and autoradiography.

#### DNA Synthetic Activity

In two normal and seven pairs of normal and vWD pigs, a 1-cm segment of the injured coronary artery and a similar segment from a noninjured coronary artery were minced, solubilized, and digested at 56°C in a solution of 2% sodium-N-lauroyl sarcosinate, 10 mM EDTA, 10 mM Tris, pH 8.0 containing 1.66 mg/ml Proteinase K (EM Biochemicals, Cincinnati, Ohio). After digestion was complete, 1.20–1.24 g CsCl was added per ml of digest to give a refractive index between 1.4001 and 1.4005. Digests were centrifuged for 66 hours at 35,000 rpm using an SW 40 rotor in a Beckman L5-65 ultracentrifuge. CsCl gradients were collected as 1–2 ml fractions and were monitored at 260 nm to identify DNA-containing fractions. DNA-containing fractions were dialyzed against 10 mM EDTA, 10 mM Tris, pH 7.4. The cpm $^3$H-thymidine/μmol DNA was determined from a
combination of liquid scintillation counting and the spectrophotometric method of Burton \(^\text{17}\) for DNA determination. \(^3\text{H}\)-thymidine uptake was expressed as:

\[
\text{cpm } \frac{\text{\(^3\text{H}\)-thymidine}}{\text{\(^3\text{H}\)-thymidine/\(\mu\text{mol DNA of balanced segment}\)}} \div \frac{\text{\(^3\text{H}\)-thymidine}}{\text{\(^3\text{H}\)-thymidine/\(\mu\text{mol DNA of control}\)}}
\]

**Autoradiography**

In an additional 3 pairs of pigs, SMC proliferation was evaluated by autoradiography. Data from one of the 3 normal pigs in this group was excluded from the study because the intravenous line infiltrated during \(^3\text{H}\)-thymidine injection. Coronary arteries were divided into 4 mm step segments and processed for histology. Autoradiography on cross sections from the proximal end of each segment was performed essentially as described by Kopriwa and LeBlond.\(^\text{18}\) The percentage of labeled SMC in each cross section was determined using a 40X objective and a 10X eyepiece with ocular grid. The percentage of labeled SMC is reported as the average of values obtained by two observers.

**Statistical Analysis**

Differences between the two phenotypes in age, weight, number of balloon passes, and \(^3\text{H}\)-thymidine incorporation by injured and control arteries were analyzed by the nonpaired, nonparametric Wilcoxon rank sum test. (Although experiments were performed in pairs, correlation analysis showed pairing to be irrelevant; therefore, a nonpaired statistical comparison was made.) The effect of the variables, phenotype, age, weight, number of balloon passes, and \(^3\text{H}\)-thymidine incorporation by control vessels on \(^3\text{H}\)-thymidine incorporation in balloonated/control vessels was evaluated by linear regression analysis.

**Results**

The uptake of \(^3\text{H}\)-thymidine in medial and intimal smooth muscle cells of coronary arteries in response to acute injury was first measured in two normal swine. The LAD of one animal was injured with three passages of the inflated balloon catheter. The control pig underwent the standard surgical procedure without balloon injury. The amount of \(^3\text{H}\)-thymidine in the DNA isolated from the balloon-injured LAD was approximately 9 times that found in the DNA from the noninjured right coronary artery (RCA) from the same animal. On the other hand, the uptake of \(^3\text{H}\)-thymidine by both the LAD and RCA from the sham-operated control was similar to the uptake of \(^3\text{H}\)-thymidine by the noninjured RCA of the pig subjected to the ballooning procedure (Figure 2).

We then compared the \(^3\text{H}\)-thymidine incorporated into DNA isolated from the injured coronary arteries of vWD and normal swine (Table 2, Figure 3). The vWD animals did not differ significantly from normal animals in age, weight, or number of balloon passes.
Figure 2. The effect of balloon catheter-induced injury on \(^3\)H-thymidine incorporation by coronary artery smooth muscle cells of normal swine. Each pig received 0.5 mCi \(^3\)H-thymidine/kg body weight 48 hours after balloon injury. In this experiment, pigs were sacrificed following a 30-minute pulse label.

Figure 3. Comparison of \(^3\)H-thymidine uptake in injured coronary arteries of normal and vWD swine. \(^3\)H-thymidine uptake is expressed as the ratio of cpm \(^3\)H-thymidine/\(\mu\) mole DNA of ballooned vessel segment to the mean cpm \(^3\)H-thymidine/\(\mu\) mole DNA of control vessel segments. Each dot represents the value obtained for a single animal. In each animal the injured artery was compared to uninjured arteries from the same animal, so each pig served as its own control.

Table 2. DNA Synthetic Activity in Injured Coronary Arteries

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Age (wks)</th>
<th>Weight (kg)</th>
<th>Balloon passes (no.)</th>
<th>cpm (^3)H-thymidine (\times 10^{-4}/\mu)mol DNA</th>
<th>(^3)H-thymidine uptake (ballooned/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWD</td>
<td>16</td>
<td>20</td>
<td>3</td>
<td>5.3*</td>
<td>1.7</td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td>20</td>
<td>3</td>
<td>8.7*</td>
<td>2.8</td>
</tr>
<tr>
<td>vWD</td>
<td>24</td>
<td>27</td>
<td>3</td>
<td>50.0*</td>
<td>21.7</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>21</td>
<td>1</td>
<td>14.0*</td>
<td>13.3</td>
</tr>
<tr>
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<td>21</td>
<td>24</td>
<td>2</td>
<td>19.0*</td>
<td>3.7</td>
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<tr>
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<td>3</td>
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<td>30</td>
<td>2</td>
<td>73.0*</td>
<td>24.7</td>
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<tr>
<td>N</td>
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<td>31</td>
<td>3</td>
<td>55.0*</td>
<td>26.2</td>
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<tr>
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<td>15</td>
<td>3</td>
<td>16.4*</td>
<td>25.2</td>
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<td>22</td>
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<td>12</td>
<td>2</td>
<td>57.5*</td>
<td>7.8</td>
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<td>18</td>
<td>20</td>
<td>3</td>
<td>0.5</td>
<td>8.9</td>
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<tr>
<td>vWD</td>
<td>12</td>
<td>12</td>
<td>3</td>
<td>52.3*</td>
<td>23.2</td>
</tr>
<tr>
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<td>18</td>
<td>27</td>
<td>1</td>
<td>3.3</td>
<td>1.2</td>
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</tbody>
</table>

*Ballooned vessel. vWD = von Willebrand's disease; N = normal.
used to induce injury \((p > 0.25, \text{Wilcoxon rank sum test})\). There was no difference between phenotypes in \(^{3}H\)-thymidine incorporation by noninjured arteries, suggesting that vWD pigs were not intrinsically different from the normal controls \((p > 0.25, \text{Wilcoxon rank sum test})\). The mean increase in the ratio of \(^{3}H\)-thymidine incorporation in balloonized/control vessels was 15.43 \((\pm 10.53 \text{ SD})\) for vWD pigs and 8.53 \((\pm 8.88 \text{ SD})\) for normal pigs. In each animal, regardless of phenotype, the uptake of \(^{3}H\)-thymidine was greatest in the DNA isolated from the injured artery, with values ranging from 1.2 to 26.2 times the uptake in nonballoonized control vessels from the same animal. The difference in incorporation of \(^{3}H\)-thymidine by balloonized vessels/control vessels between the two phenotypes was not statistically significant \((\text{Wilcoxon rank sum test}, p > 0.25)\). When the ratio of \(^{3}H\)-thymidine incorporation in balloonized/control arteries was analyzed as a function of phenotype, age, weight, number of balloon passes, and \(^{3}H\)-thymidine incorporation by noninjured vessels, none of the variables was found to have a significant influence on incorporation \((p > 0.10, \text{linear regression analysis})\). The wide range in thymidine incorporation led us to examine the proliferative response of injured vessels by autoradiography. In three pairs of pigs, we examined sections from the proximal end of 4 mm-step segments along the coronary arteries. Figure 4 is a representative autoradiograph showing thymidine incorporation into the nuclei of medial SMC. SMC labeling was not always uniform throughout each cross-section. In some cross-sections one quadrant of the vessel wall contained a higher percentage of labeled nuclei than did other quadrants in the same section. At the light microscopic level, there were no discernible differences in the degree of intimal injury between areas of heavy and light labeling. More heavily labeled regions of the coronary arteries were not associated with branch points. Figure 5 shows the distribution of SMC labeling observed at 4-mm intervals along the injured coronary arteries of vWD and normal swine compared to a noninjured control vessel from each phenotype. There was a considerable range in the extent of labeling in both phenotypes. However, no differences between phenotypes were documented.

**Figure 4.** Autoradiograph of a coronary artery 48 hours after endothelial injury showing \(^{3}H\)-thymidine incorporation into the nuclei of medial smooth muscle cells. Bar = 10 \(\mu\text{m}\).
Our recent morphological data suggested that at 30 minutes after injury, platelet activation is impaired at sites of superficial injury in coronary arteries of swine with vWD; however, the extent to which these observations reflect abnormalities in the release of PDGF is not known. Although platelets adherent to the exposed subendothelium in swine with vWD may never, or only later, achieve maximum release of PDGF, the amount of PDGF released may be sufficient to initiate the proliferative response.

In vitro studies have shown that the effect of vWF on platelet adhesion is shear-dependent. However, it is unlikely that the stasis associated with the ballooning procedure may have compromised the ability of our model to detect differences between phenotypes, since our previously published studies of morphological evidence of impaired platelet activation in vWD swine used this same model of acute injury.

Alternatively, the proliferative response to injury that we have observed in vWD swine may be due to factors other than PDGF. A variety of other substances such as insulin, lipoproteins, macrophage-derived growth factor, and endothelial cell-derived growth factor that promote growth of SMC and related connective tissue constituents have been described. Although all of these substances may function to stimulate SMC replication at some time during plaque formation, it is more likely that the platelet or endothelial cell is the cell type responsible for the primary stimulus. Gajdusek et al. have recently isolated from endothelial cell-conditioned media a polypeptide that is a potent stimulator of DNA synthesis in cultured bovine aortic smooth muscle cells. Of particular interest is the preliminary report that supernatant from freeze-thawed porcine aortic endothelial cells, from both normal and vWD pigs, stimulated a 30-fold increase in DNA synthesis in cultured mouse 3T3 cells. When compared with a crude preparation of porcine platelet-derived protein, this endothelial cell extract was tenfold as potent a mitogen.

The reason for the variability in SMC responses seen in both normal and vWD pigs is not clear. In this model, SMC proliferation does not appear to be a function of the age or weight of the animal or of the number of balloon passes used to induce injury (p > 0.10). By limiting the amount of air used to inflate the balloon at the catheter tip to 0.3 ml air, deep injury characterized by a disruption of the internal elastic lamina and medial involvement has been eliminated. However, more subtle differences in the milder form of endothelial injury may exist. The effect of altering the luminal surface of the artery on rheological phenomenon is not known. Turbulence or stasis created by the injury could cause the concentration of growth regulators to vary at different points along the vessel wall. Since not every vascular insult progresses to an atherosclerotic plaque, there must be mechanisms for preventing or modulating the SMC response. Extracts of aortic tissue reportedly inhibit SMC prolif-

**Figure 5.** Distribution of smooth muscle cell (SMC) labeling observed at 4 mm intervals along injured arteries of normal and vWD swine. Autoradiography was performed on cross sections from the proximal end of each segment. Each dot represents the percentage of labeled SMC nuclei in cross-sections from a single segment. Values obtained for a noninjured control vessel from each phenotype are shown for comparison.

**Discussion**

We have compared the effects of balloon catheter-induced injury on the proliferative response of SMC in coronary arteries of normal swine and swine with vWD. The results show that SMC are stimulated to synthesize DNA in response to acute injury in both normal and vWD swine. The magnitude of the response, although variable, appears to be unrelated to phenotype.

The amount of platelet-derived growth factor (PDGF) required for in vivo stimulation of SMC proliferation and the particular circumstances under which SMC are receptive to stimulation are not known. Using an indirect immunofluorescent technique, Goldberg and co-workers have provided evidence that platelet factor 4 (PF4), a platelet-specific protein closely related to PDGF, penetrates the vessel wall within minutes following endothelial injury. Their studies have suggested that the time span during which platelets release PF4 and the vessel wall is permeable to it is short, since immunofluorescence within the vessel wall is virtually undetectable 4 hours after injury. In rabbits, aortic endothelial denudation is followed by a 24-hour latent period in SMC DNA synthesis with a peak in synthetic activity occurring 48 hours after injury. Without further injury, the proliferative process appears to be self-limiting.
vation in swine, and a heparin-like substance produced by bovine endothelium also appears to inhibit SMC growth. Thus, the variability of SMC response may reflect the extent of involvement of promotors versus inhibitors of SMC growth.

Our present studies showing no difference in the proliferative response of SMC to acute injury in the coronary arteries of normal versus VWD swine are supportive of our previous study showing a similar degree of atherosclerotic plaque formation in the coronaries of both phenotypes. However, our model of acute injury differs from the more chronic process thought to occur in the development of human atherosclerosis. Therefore, these studies do not exclude the possibility of detecting differences in SMC response between normal and VWD pigs using models of more chronic forms of injury. There is some evidence for a regional variation in SMC response to injury. Scott et al. have identified “intimal cell cushions” in the abdominal aorta of swine where lesions predominate, and Goldberg et al. have presented data suggesting that, in the rabbit, the response of abdominal aortic SMC to intimal injury is greater than that in the thoracic aorta. Since VWD swine appear to show a limited resistance to atherosclerosis in the thoracic aorta, it may be that the influence of VWF on atherogenesis is subject to regional variations in smooth muscle cell growth kinetics.

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References


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