Platelet Inhibition Reduces Cyclic Flow Variations and Neointimal Proliferation in Normal and Hypercholesterolemic-Atherosclerotic Canine Coronary Arteries

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Background—Platelet-derived growth factors help stimulate the neointimal proliferation of restenosis after coronary interventions. Reducing platelet accumulation at treated sites may attenuate restenosis. We tested this hypothesis by inducing repetitive platelet aggregation at coronary angioplasty sites in dogs and measuring subsequent neointima formation.

Methods and Results—Cholesterol-sensitive dogs (n = 74) received either 4% cholesterol-enriched diets for >8 months (n = 29), creating visible atheromas, or normal canine diets (n = 45). A coronary balloon angioplasty cyclic flow variation (CFV) model was used. One group of control dogs (group 1, n = 8) had angioplasty with no arterial constriction applied and no drug treatment. Three other groups had arterial constrictors applied to provoke CFVs: group 2 (n = 28) received no drug therapy, group 3 (n = 18) received oral aspirin alone, and group 4 (n = 20) received 3 oral antplatelet agents: ridogrel, ketanserin, and clopidogrel (R + K + C) to simultaneously inhibit the thromboxane A2, serotonin, and ADP pathways of platelet aggregation, respectively. Bleeding times were moderately prolonged in the aspirin-treated group (124 ± 9 seconds after 3 weeks versus 76 ± 6 seconds at baseline, P < 0.01) and greatly prolonged on R + K + C (>600 versus 104 ± 5 seconds, P < 0.001). The frequency and severity of CFVs were inversely related to the degree of platelet inhibition and prolongation of bleeding times, as was sudden death due to acute thrombotic coronary occlusion. Quantitative histology at 8 weeks revealed increased intima-to-media ratio with CFVs: 0.89 ± 0.14 in the untreated group 2 versus 0.11 ± 0.04 in the control group (P < 0.001). Intima-to-media ratio was significantly reduced with antplatelet treatment (0.27 ± 0.05 with aspirin treatment and 0.20 ± 0.05 with R + K + C treatment, respectively, P < 0.001). Cholesterol feeding did not appear to influence results.

Conclusions—Repetitive platelet accumulation at coronary angioplasty sites caused enhanced neointimal proliferation by 8 weeks. Oral inhibitors of platelet aggregation attenuated platelet function, prolonged bleeding times, reduced or prevented cyclic flows and abrupt thrombotic occlusions, and thereby inhibited neointimal proliferation. Platelet inhibition should continue to receive attention in efforts to reduce restenosis after coronary interventions. (Circulation. 2001;104:2331-2337.)

Key Words: platelets ▪ blood flow ▪ hypercholesterolemia ▪ atherosclerosis ▪ arteries

Restenosis after coronary interventions involves new tissue growth at treated arterial sites. Medial and adventitial smooth muscle cells and fibroblasts participate in this, overgrowing the disrupted area and synthesizing extracellular matrix, thereby forming a neointima.1, 2 Evidence from animal models and human atherectomy specimens indicates that peptide growth factors released from platelets stimulate medial smooth muscle cells after interventions.3– 5 In addition, the nonpeptide platelet mediators thromboxane A2 (TXA2), serotonin (5-HT2), and ADP have recently been shown to possess their own mitogenic effects, amplifying smooth muscle cell proliferative responses both to growth factors and to one another.5– 8 Additional amplification occurs when low-density lipoproteins (LDLs) are elevated.9 One important and relevant model of platelet accumulation at coronary angioplasty sites is the cyclic flow model developed by Folts et al.10 In this model, waves of fresh platelets aggregate and dislodge recurrently, creating characteristic cycles in flow. These cyclic flow variations (CFVs) provide continuous mediator delivery to injured arterial sites. We used this model to prospectively test the

Received February 1, 2001; revision received August 24, 2001; accepted August 29, 2001.
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Guest Editor for this article was Elliott M. Antman, MD, Brigham and Women’s Hospital, Boston, Mass.
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hypothesis that CFVs enhance neointimal proliferation after coronary angioplasty, whereas oral platelet inhibition with antagonists to TxA₂, 5-HT₂, and ADP together attenuate it.

Methods

Animal Model and Surgical Preparation

The model we used has been described previously. The present studies were performed in 77 dogs weighing 30 to 35 kg, selected from a colony of cholesterol-sensitive breeding dogs. Pilot studies indicated that prolonged cholesterol feeding raised serum cholesterol values to 800 to 1000 mg/dL. The remaining 45 dogs consumed regular canine chow. Each dog had been monitored for 30 minutes to confirm mild CFVs and ensure stability and the absence of immediate occlusions, flow probe wires were exteriorized through the thorax. Chests were closed, and animals were allowed to recover. Dogs were vents with reinforced tethers and swivels, permitting connection to electronic flow monitors. Monitoring was continuous for 21 days, then briefly every week for weeks 4 through 8. Surviving dogs were killed by sodium pentobarbital overdose 8 weeks after surgery.

Antiplatelet Therapy

Four groups were created (Table 1). Group 1 had coronary angioplasty only, without coronary constrictors or drug therapy. Groups 2, 3, and 4 all had coronary constrictors placed after angioplasty. Group 2 dogs did not receive drugs (No Tx). Group 3 dogs received only oral aspirin (ASA) 325 mg/d. Group 4 dogs received 3 antiplatelet agents (R+K+C orally: ridogrel (a thromboxane A₂ synthase inhibitor and receptor antagonist), 400 mg/d the first week, 800 mg/d the second week, and 1.2 g/d 3 weeks and beyond; ketanserin (a 5-HT₄ receptor antagonist), 500 mg/d; and clopidogrel (an ADP antagonist), 500 mg/d. Doses were based on pilot studies confirming inhibition to a thromboxane mimetic (U46619), 5-HT, and ADP.

Drug-treated dogs (groups 3 and 4) began therapy 3 weeks before surgery and continued it for 3 weeks afterward (total 6 weeks treatment). All dogs in groups 1, 2, and 3 received heparin 100 U/kg IV at the start of the procedures. In group 4 (R+K+C group), the first 3 dogs received heparin at the start but had fatal internal bleeding later after thoracotomy closure. Heparin was thereafter eliminated from procedures in the remaining group 4 dogs, and no further abnormal bleeding occurred.

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**TABLE 1. Animal Groups, Treatments, and Survival**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Constrictor Placed</th>
<th>Drug Treatment</th>
<th>Subgroup</th>
<th>Diet</th>
<th>Died Suddenly During Monitoring, n (%)</th>
<th>Survived 8 Weeks, n (%)</th>
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</thead>
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<td>8</td>
<td>No</td>
<td>None</td>
<td>...</td>
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<td>7 (87)</td>
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<td>Normal</td>
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<td>11 (65)</td>
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<td></td>
<td></td>
<td></td>
<td>2-A (n=17)</td>
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<td>12 (43)</td>
<td>16 (57)</td>
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<td></td>
<td></td>
<td></td>
<td>2-B (n=11)</td>
<td>Chol</td>
<td>6 (55)</td>
<td>5 (45)</td>
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<tr>
<td>3</td>
<td>18</td>
<td>Yes</td>
<td>ASA</td>
<td>All</td>
<td>Normal</td>
<td>2 (11)</td>
<td>16 (89)</td>
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<td></td>
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<td>1 (10)</td>
<td>9 (90)</td>
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<tr>
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<td></td>
<td></td>
<td></td>
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<td>Chol</td>
<td>1 (13)</td>
<td>7 (87)</td>
</tr>
<tr>
<td>4</td>
<td>20*</td>
<td>Yes</td>
<td>R+K+C</td>
<td>All</td>
<td>...</td>
<td>0</td>
<td>20 (100)</td>
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<td>Normal</td>
<td>0</td>
<td>10 (100)</td>
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<td></td>
<td>4-B (n=10)</td>
<td>Chol</td>
<td>0</td>
<td>10 (100)</td>
</tr>
</tbody>
</table>

Chol indicates cholesterol.

*Three additional dogs had fatal bleeding before heparin was discontinued.
Hematology Studies
Blood samples for hematocrits, platelet counts, bleeding times, and platelet aggregation studies were obtained (1) before the beginning of drug therapy, (2) just before surgery, (3) 3 weeks after surgery (conclusion of drug therapy in treated groups), and (4) just before death at 8 weeks. Bleeding times were measured with the Simplex 2 cutaneous method. Platelet aggregation was measured by electrical impedance (Chronolog) with 3 agonists: U46619, 5-HT, and ADP.

Histology
Thoracic aortas were cannulated immediately after death. Hearts were pressure-perfused and fixed in situ with neutral buffered formalin. Segments of LADs were dissected free and embedded in paraffin. Multiple sections were made, stained with Verhoeff–Van Gieson and Movat stains, and examined under light microscopy. With a video camera with imaging screen and computer-linked digitizing pad, borders of vessel lumens and internal and external elastic laminae were traced. Average values for 3 to 5 sections were calculated and used as representative measurements. Percentage lumen area stenosis was calculated as \(\{1-\text{[lumen area/lumen area+intimal area]}}\) × 100%. This variable and the ratio of intima to media (I/M ratio) were of primary interest.

Statistical Analyses
Data are presented as mean ± SEM unless otherwise noted. Comparisons between groups were performed with ANOVA and nonparametric Kruskal-Wallis rank order tests. Repeated-measures ANOVA was used to compare hematological and coagulation data on dogs within the same group. A value of \(P\leq 0.05\) was considered significant.

Results
Hematology, Coagulation, and Platelet Aggregation
Measured variables are shown in Tables 2 and 3. Hematocrits fell slightly but significantly during drug treatment in group 4 dogs only, whereas platelet counts rose in this group (Table 2). These changes did not appear to be clinically meaningful. Activated clotting times, prothrombin times, and activated partial thromboplastin times were not significantly different in any group (data not shown). Platelet aggregation indices and bleeding times, however, were markedly different (Table 3). In untreated group 2 dogs, values were unchanged,
whereas in group 3 (ASA treatment), average platelet aggregation responses to U46619, 5-HT3, and ADP were all slightly but significantly reduced while on therapy, and in group 4 (R+K+C treatment), platelet aggregation was abolished on therapy. Bleeding times in group 3 increased from baseline to procedure (124±9 versus 76±6 seconds, \(P<0.01\)), recovered some at 3 weeks (92±6 seconds), and returned to baseline by 8 weeks. Platelet aggregation responses were concordant in direction and magnitude with changes in bleeding times.

**Cyclic Flows and Survival**

During chronic monitoring, flows in LADs were recorded continuously for 3 weeks. The number of CFVs with mild (nadir flow 75% to 100% of baseline), moderate (nadir flow 25% to 75% of baseline), and severe (nadir flow <25% of baseline) flow reductions or persistent low-flow states were counted (Figure 1). There was an association between the number and severity of CFVs recorded during monitoring and survival in the 4 groups (Figure 2 and Table 1). Six of the 8 dogs in group 1 developed CFVs during monitoring, and 1 died of abrupt occlusion of the artery (1/8 = 13%). There were 28 dogs initially in group 2; 12 (43%) died during chronic monitoring, leaving 16 dogs surviving 8 weeks (I/M = 0.213 versus group 1). There were 18 dogs initially in group 3; 2 (11%) died during chronic monitoring, and 16 survived 8 weeks (I/M = 0.222 versus group 2). Of 23 dogs initially in group 4, the first 3 dogs (treated with heparin) died of excessive bleeding after their procedures. Bleeding occurred within the thoracotomy wound during the first 24 hours and was uniformly fatal. Heparin was thereafter eliminated from the angioplasty protocol for group 4 dogs only, and the remaining 20 dogs in this group were treated with the 3 antiplatelet agents without heparin. No further bleeding problems occurred, and these dogs all had uncomplicated postprocedure courses; none died during the 8-week period (\(P=0.218\) versus group 3). Furthermore, none of the group 4 dogs developed CFVs, despite attempts with more severe arterial constrictions after angioplasty to produce them. In groups 2 and 3, the constrictions applied to produce CFVs reduced average flow velocities to 83±4% and 86±6% of baseline values, respectively. In group 4, the constrictions after angioplasty were increased stepwise until average flow velocities were reduced to 18±2% of baseline, and CFVs still were not produced, nor did any occlusions occur then or later.

**Neointimal Proliferation**

The amount of neointima was significantly greater by quantitative histology in group 2 dogs (with uninhibited CFVs) than in group 1 angioplasty-only dogs with few CFVs (I/M ratio 0.89±0.14 versus 0.11±0.04, \(P<0.0001\); Table 4, Figure 3). Reduction or prevention of CFVs in groups 3 and 4 greatly reduced neointimal proliferation seen at 8 weeks. In group 3, the average I/M ratio was reduced by 70%, from 0.89±0.14 to 0.27±0.05 (\(P<0.001\)). In group 4, a further 20% reduction in average I/M ratio was found (0.20±0.05 versus 0.27±0.05, \(P=0.33\)). Similar results were seen for the percent lumen area stenosis.

**Discussion**

This study reaffirms the observation that CFVs after coronary angioplasty are associated with extensive neointimal proliferation. Cyclic flows are platelet-mediated and occur spontaneously with plaque ulceration or fissuring or after angioplasty.\(^{12-14}\) Curtailing CFVs with orally administered platelet inhibitors attenuates the amplified neointimal response. This was seen even when profound hyperlipidemia coexisted. Our findings provide further evidence that platelet factors underlie neointimal proliferation in restenosis. In a previous retrospec-
Platelets and Restenosis

Activated platelets release peptide growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor, transforming growth factor-β, and possibly others. Abe and coworkers\(^9\) found increased PDGF receptors in medial smooth muscle cells of balloon-injured rat carotid arteries, priming them for platelet stimulation after angioplasty. Blockade of PDGF receptors was noted to inhibit neointimal growth after balloon injury in baboons.\(^{17}\) Fundamentally, the total amount of PDGF released locally can be reduced if platelet accumulation is reduced at angioplasty sites. For example, directly inhibiting platelet adhesion by targeting specific platelet membrane adhesion receptors was found to reduce neointima formation in several experimental models.\(^{18-20}\)

It was recently discovered that TxA\(_2\), 5-HT\(_2\), and ADP, nonpeptide substances previously thought to be involved only in platelet activation, can in addition stimulate smooth muscle cell growth on their own. Pakala and coworkers\(^8\) reported dose-related mitogenic stimulation of smooth muscle cells by TxA\(_2\) and 5-HT\(_2\) at nanomolar concentrations. These effects were blocked by specific antagonists to TxA\(_2\) and 5-HT\(_2\) receptors. Crowley and colleagues\(^6\) found that growth amplification of smooth muscle cells in vitro by 5-HT\(_2\) and ADP was greatest at physiologically relevant concentrations of peptide growth factors. Furthermore, proliferation of smooth muscle cells in culture was inhibited 35% to 45% by the 5-HT\(_2\) antagonists ketanserin and LY53857, as well as the ADP antagonist apyrase. We previously reported that ketanserin, LY53857, and apyrase inhibit platelets and prevent CFVs in our canine model.\(^{21-23}\) Matsuno and colleagues\(^{24}\) likewise reported reductions in neointima formation proportional to the degree of inhibition of platelet aggregation to ADP. It is therefore attractive to hypothesize that inhibiting platelet accumulation and thrombus formation at angioplasty sites may attenuate neointima development in 2 ways: first, by reducing the total number of platelets deposited, which would reduce growth factor delivery, and second, by blocking the amplification signals that may occur from whatever TxA\(_2\), 5-HT\(_2\), and ADP are released.

Lipids

In vitro studies indicate that vascular smooth muscle cell proliferation (cell number) is twice as great at 24 hours when high concentrations of LDL or oxidized LDL exist in the presence of 5-HT\(_2\).\(^9\) A linkage also exists between hyperlipidemia-induced atherosclerosis and thrombosis in several animal models and in humans.\(^{25,26}\) From such knowledge, one of our initial hypotheses was that sustained hyperlipidemia would be associated with more profound neointima formation after angioplasty, and platelet inhibition in that setting would provide greater benefits. We did not, however, find significant differences in neointima after angioplasty between cholesterol-fed and normal diet-fed dogs. The reasons for this are not clear and require further study. By visual examination, fatty streaks and plaques were confined exclusively to the cholesterol-fed dogs, representing a type of diffuse atherosclerotic process (Figure 1). To the best of our knowledge, this is the first time a hyperlipidemia-driven atherosclerotic dog model has been used. In rabbit models, by way of contrast, hyperlipidemia is associated with enhanced neointima after angioplasty, and lipid lowering with statins attenuates it, predominantly by reducing both macrophage infiltration and matrix metalloproteinase expression.\(^{27,29}\) It is possible that in this canine model with exaggerated platelet effects (CFVs), the cell kinetics may be different, and the neointimal proliferative response at a single angioplasty site is only weakly affected by the high lipid levels.

Cyclic Flow Model

CFVs result from repetitive accumulation and dislodgment of platelet aggregates along with dynamic vasoconstriction at sites of coronary stenosis with endothelial injury.\(^{12}\) Abrupt thrombotic occlusion, infarction, malignant arrhythmias, and death are all potential consequences of CFVs in coronary arteries, paralleling human clinical events and making this a useful model.\(^{14,30}\) In the present study, aspirin reduced the number of thrombotic occlusions as well as total CFV count over 3 weeks, although not as much as the 3-drug combination did. Aspirin had slight but significant effects on platelet aggregation indices and bleeding times, yet these changes were accompanied by significant lessening of neointimal proliferation. More intense inhibition of TxA\(_2\), 5-HT\(_2\), and ADP, which prevented CFVs entirely, had smaller additional...
inhibitory effects on neointimal growth. It is important to note here, however, that the monitored variable, CFVs, require provocation by arterial constriction. Usually only minor constriction is needed to produce CFVs after angioplasty (≈75% of preconstrictor flow velocity).\textsuperscript{12} In the group 4 dogs here, however, we constricted coronary flows much more severely, down to levels that would have produced immediate thrombosis if intense platelet antagonism had not been used. Without intense platelet inhibition, such constriction would not otherwise have been possible. Therefore, the additional neointimal growth reduction we observed occurred in a setting of much more profound stimulus to CVF development.

Although vascular remodeling may occur after angioplasty, with changes in arterial dimensions due to enlargement or shrinkage of the wall, the degree to which this contributes to restenosis is not precisely known.\textsuperscript{31,32} Whether vascular remodeling itself is model-dependent is uncertain.\textsuperscript{33,34} For example, in diabetics\textsuperscript{35} and with stents,\textsuperscript{36} restenosis is predominantly if not exclusively due to neointimal growth. Using dogs in this well-defined model system, we found that the dimensions of both the internal and external elastic laminae were not significantly different at 8 weeks between the 4 groups. This suggests that remodeling did not occur to any great extent in this canine model and probably does not explain our findings.

Limitations
Without dose-response relationships for platelet activation indices and new tissue growth, it is not possible to know precisely how to administer oral platelet inhibitors for this purpose. Growth factors were not measured directly in tissue samples, nor were nonpeptide mediator levels measured directly. Instead, we relied on relevant aggregation indices as indirect measures of growth factor inhibition. In addition, the duration of treatment (and pretreatment) with the oral agents and the timing of histological examination were entirely empirical.

The first 3 dogs in group 4 had fatal bleeding after thoracotomy, leading to discontinuation of procedural heparin in that group. After heparin was omitted, no group 4 dogs had bleeding problems, which is remarkable, because bleeding times were uniformly very high (>600 seconds). This suggests that remaining platelet activation pathways (thrombin in particular) provided sufficient hemostasis after thoracotomy. Relatively little is known about interactions of platelet antagonists and heparin in the generation of bleeding. Evidence from recent clinical trials suggests a relationship,\textsuperscript{37} and when platelet antagonism is used, the therapeutic window with concomitant heparin therapy is narrow. This has translated in clinical practice to weight adjustment of heparin along with overall reductions in heparin dose.\textsuperscript{38}

Conclusions
CFVs after coronary angioplasty were associated with extensive neointimal proliferation at 8 weeks in normal and atherosclerotic canine arteries. Platelet inhibition with 3 specific oral agents directed against the nonpeptide platelet mediators TxA\textsubscript{2}, 5-HT\textsubscript{3}, and ADP, when administered for 3 weeks before until 3 weeks after angioplasty, eliminated cyclic flows and greatly reduced this neointimal proliferation. Aspirin by itself had significant effects in this model, albeit under less provocative conditions than the combination drugs. Because neointimal proliferation appears to be linked to platelets and platelet-derived mediators, we believe platelets should continue to receive widespread attention in efforts to reduce restenosis.

Acknowledgments
This study was supported in part by grants from the NIH, NHLBI (RO-1-HL-54839); the Gordon Cain Foundation; and the State of Texas.

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_Circulation_. 2001;104:2331-2337
doi: 10.1161/hc4401.098434

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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