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Frequency and severity of cyclic flow alternations and platelet aggregation predict the severity of neointimal proliferation following experimental coronary stenosis and endothelial injury

(thromboxane A_2/serotonin/restenosis)

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ABSTRACT **The role of recurrent platelet aggregation in the development of neointimal proliferation of coronary arteries was explored in this study, and the hypothesis was evaluated that recurrent platelet aggregation and the consequent frequency and severity of cyclic coronary blood flow variations are important pathophysiologic factors in the subsequent development of neointimal proliferation. In 24 chronically instrumented dogs, variable degrees of coronary artery neointimal proliferation were observed 3 weeks after mechanical injury of the arterial endothelium and the placement of an external coronary artery constrictor. The severity of neointimal proliferation at 21 days was closely related to the frequency and severity of cyclic coronary blood flow variations during the initial 7 days after instrumentation of the animals, itself a manifestation of recurrent platelet aggregation and dislodgement. Pharmacological therapy with a dual thromboxane A_2 synthetase inhibitor and receptor antagonist and with a serotonin S_2 receptor antagonist frequently was successful in abolishing cyclic blood flow variations and in retarding neointimal proliferation.**

Acute coronary artery disease syndromes, including unstable angina pectoris and acute myocardial infarction, may be caused by the accumulation of platelet aggregates at sites of coronary artery stenosis and endothelial injury (1–4). Aggregating platelets release mediators that may promote further platelet aggregation, dynamic coronary artery vasoconstriction, and neointimal proliferation in the arterial wall (1, 5–11). When coronary artery angioplasty is used as a treatment for focal atherosclerotic lesions, injury to the endothelium and media of the arterial wall occurs, and platelet aggregates form at these sites (12–16). It has been postulated that platelet aggregates accumulating at angioplasty sites may release factors that mediate a fibroproliferative response (1, 3, 15–18). A proliferative response of smooth muscle cells in the media and intima has been shown to cause the restenosis phenomenon after angioplasty (12–14, 18). In this report, we present evidence suggesting a strong association between the frequency and severity of platelet aggregation, as detected by cyclic alterations in coronary blood flow, thrombosis, and neointimal proliferation following endothelial injury at sites of experimentally created coronary artery stenoses in a canine model. We also demonstrate that a potent antiplatelet regimen consisting of a combined thromboxane synthesis inhibitor and receptor antagonist and of a serotonin receptor antagonist given continuously for 15 days usually markedly attenuates the neointimal proliferation in this experimental model.

MATERIALS AND METHODS

The experiments were performed in mongrel dogs. Anesthetized animals had a thoracotomy under sterile conditions. The left anterior descending (LAD) coronary artery was gently dissected free of surrounding tissues. A cylindrical pulsed Doppler flow probe was placed around the exposed portion of the LAD and continuous recordings of LAD blood flow velocity were obtained. The arterial endothelium was injured by gently squeezing the external surface of the exposed artery with cushioned forceps. Next, a small plastic constrictor was placed around the artery at the site of endothelial injury. The chest was closed. After surgery, all animals were monitored closely with continuous recordings of LAD blood flow velocities and aortic blood pressures.

A total of 24 dogs were followed for 21 days. Seven of the dogs did not receive any treatment and served as controls. Another 17 dogs received the antiplatelet agents ridogrel (a dual thromboxane A_2 synthetase inhibitor and thromboxane A_2 receptor antagonist; Janssen Pharmaceuticals) (19) and either ketanserin (a serotonin S_2 receptor antagonist; Janssen Pharmaceuticals) (20) or LY53857 (another serotonin receptor antagonist; Eli Lilly) (21). Of these antiplatelet agent-treated dogs, 7 received bolus doses of ridogrel at 5 mg/kg and either ketanserin at 0.5 mg/kg or LY53857 at 0.2 mg/kg every 8–12 hr for 7–14 days through catheters positioned in the left atrium. The remaining 10 dogs received ridogrel as bolus injections (5–10 mg/kg) every 8 hr and as a continuous infusion (0.6 $mg \cdot kg^{-1} \cdot hr^{-1}$) and ketanserin as bolus injections (1–2 mg/kg) every 8 hr and as a continuous infusion (0.1–0.2 $mg \cdot kg^{-1} \cdot hr^{-1}$) for 14 days. *Ex vivo* platelet aggregation was evaluated in these animals before applying the arterial constrictor and injuring the endothelium, and everyday during the period when the treatments were given by using a modified Born's method and the agonists ADP (final concentration, 10–20 μM), U46619 (a thromboxane mimetic, at 50–100 ng/ml), and serotonin (1–2 μM). The results of *in vitro* platelet aggregation studies were used to adjust *in vivo* dosages of the thromboxane A_2 and serotonin inhibitors given by sustained infusion to ensure complete or near complete abolition of *in vitro* responses to the platelet agonists as a result of *in vivo* infusion of the combined antagonists.

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Abbreviations: LAD, left anterior descending; CFV, cyclic flow variation.

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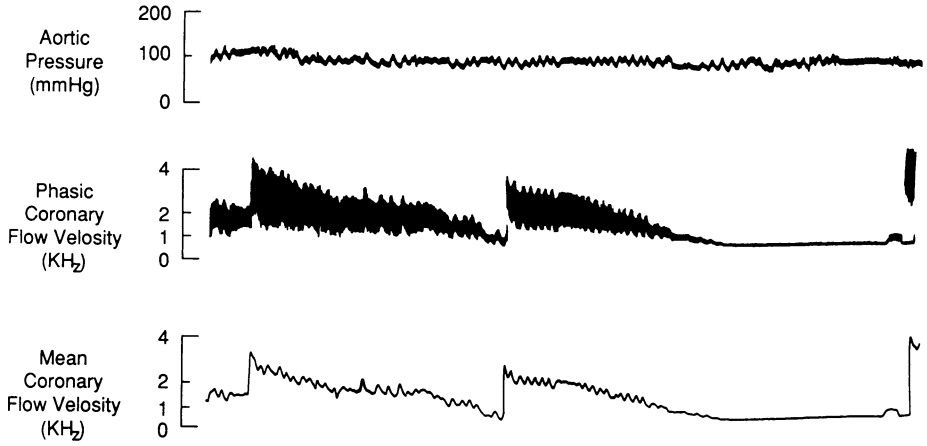

FIG. 1. Representative tracing from a dog on the 4th day after surgery. Aortic pressure was recorded from a catheter placed in the aorta. Coronary artery blood flow velocity was recorded from a pulsed Doppler flow probe externally positioned on the LAD coronary artery above a site of stenosis and endothelial injury. Coronary blood flow reductions are caused by *in vivo* platelet aggregation and dynamic vasoconstriction associated with the local accumulation of thromboxane A_2 and serotonin in this experimental model (1, 2).

After sacrifice, segments of the LAD and the circumflex coronary artery were dissected from the hearts, fixed in phosphate-buffered formalin, and embedded in paraffin. Histological sections were prepared. On photographic prints of the sections, structures were drawn onto the prints while examining the original slides by light microscopy. These areas were the media, original lumen (outlined by the inner edge of the media and delineated by the circumferential projection of the internal elastic lamella), area of intimal proliferation, and residual lumen. These areas were measured with a computer-linked digitizing tablet. The percentage stenosis was calculated as

$$\frac{1 - \text{residual lumen area}}{\text{residual lumen} + \text{intima area}} \times 100.$$

RESULTS

Cyclic Flow Variations (CFVs). Coronary artery CFVs (Fig. 1) occur in dogs with coronary artery stenosis and endothelial injury, and they correlate with repetitive platelet aggregation and dislodgement and vasoconstriction at sites of coronary artery stenosis and endothelial injury (16, 22–29). CFVs developed in 4 of the 7 nontreated dogs. CFVs also developed in 4 of the 17 dogs that received combined treatment with two antiplatelet agents, ridogrel and either ketanserin or LY53857, that have been shown to be effective in eliminating CFVs in anesthetized and awake dogs (22, 24–29). During the initial coronary flow recordings after surgery, most dogs had CFVs that disappeared in the following 12 hr. In the animals that developed CFVs later, they usually occurred 2–4 days after the surgical procedures.

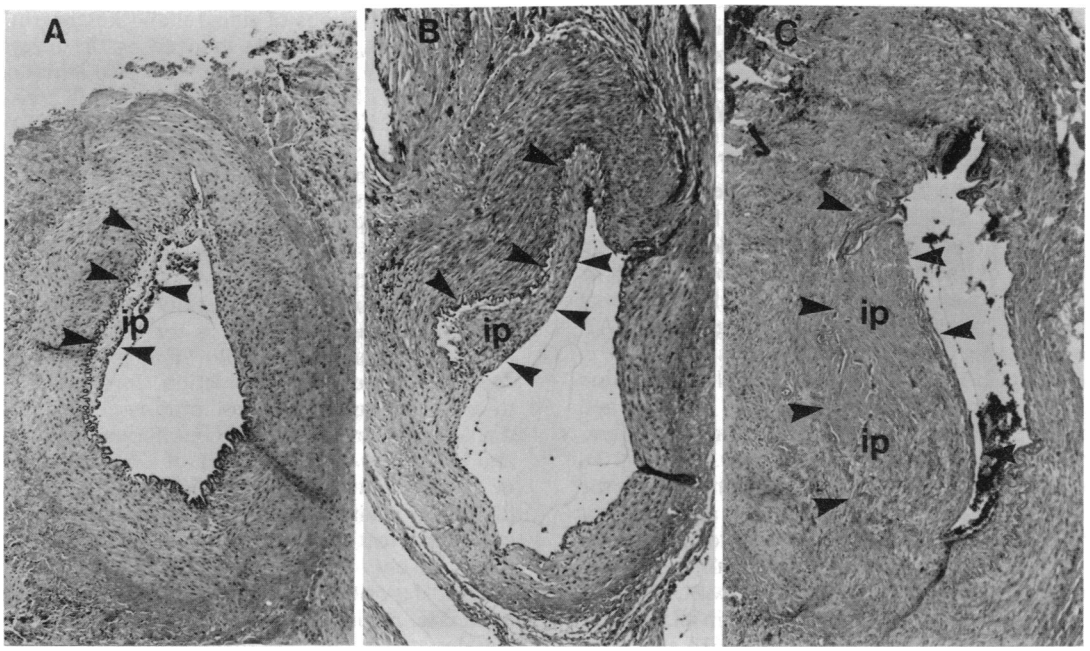

FIG. 2. Histological changes within the LAD coronary arteries 21 days after stenosis and endothelial injury. The original arterial lumen was determined by the internal elastic lamella or edge of the media where the internal elastic lamella is absent. Neointimal proliferation (ip) is the area inside the internal elastic lamella or edge of the media and is indicated by arrowheads. (*A*) LAD from a dog without CFVs shows minimal intimal proliferation. (Hematoxylin and eosin; ×45.) (*B*) LAD from a dog with frequent cyclic flow reductions in the 1st week after endothelial damage had moderately severe intimal proliferation. (Hematoxylin and eosin; ×45.) (*C*) LAD from a dog with 1321 cyclic flow reductions in the 1st week after endothelial damage has severe intimal proliferation. (Hematoxylin and eosin; ×45.)

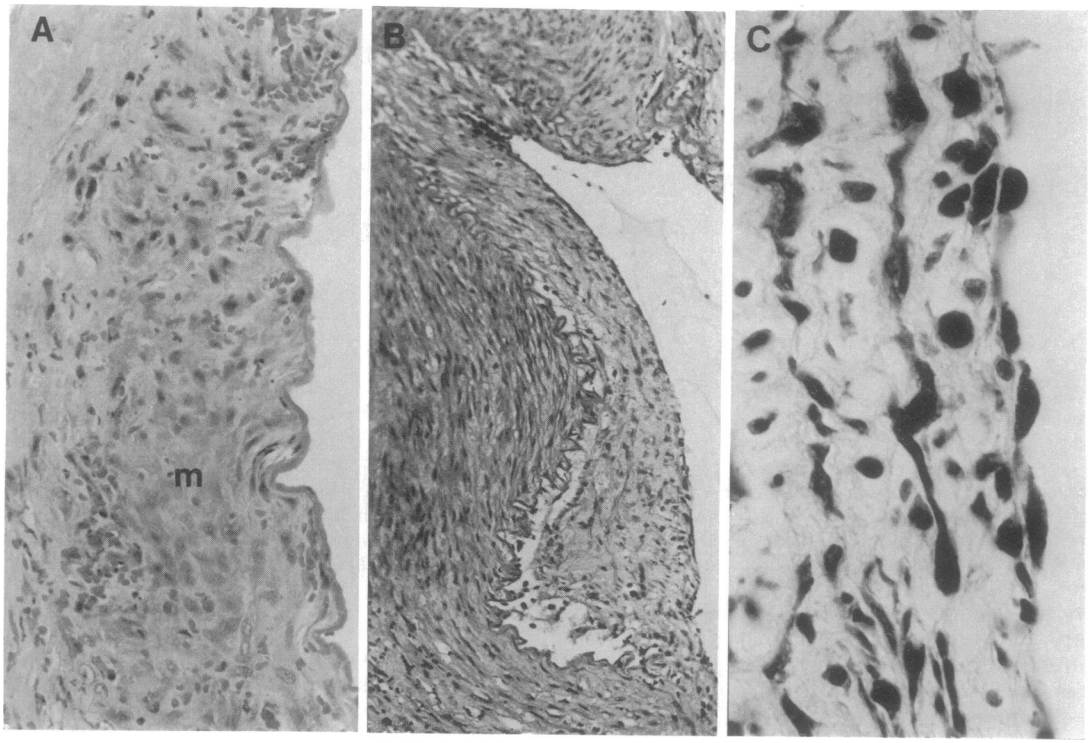

FIG. 3. Histology of the LAD early and late after endothelial injury. (*A*) At 2 days, the intimal surface overlying the media (m) is denuded of endothelium. (Hematoxylin and eosin; ×210.) (*B*) At 21 days, there is prominent neointimal proliferation. The intimal lesion is composed of a loose connective tissue containing numerous elongated cells, confirmed by electron microscopy to be modified smooth muscle cells. Endothelium is present on the surface of the lesion. (Hematoxylin and eosin; ×100.) (*C*) Higher magnification view of intimal lesion demonstrates elongated smooth muscle cells, some round leukocytes, and endothelial cells on the surface. (Movat; ×310.)

Morphologic Analyses. On the 21st day after surgery, animals were sacrificed with large doses of intravenous sodium pentobarbital. Coronary artery tissues were obtained for histological studies that revealed various degrees of neointimal proliferation with modified smooth muscle cells present in the thickened intima of the LAD at sites of endothelial injury and arterial constriction (Figs. 2 and 3). Some vessels also showed evidence of organized thrombi.

Severity of Neointimal Proliferation. The severity of neointimal proliferation was related to the frequency and severity of CFVs (Figs. 3 and 4). In the 6 dogs with severe CFVs defined as more than 9 flow reductions >70% of baseline flow velocity values during the 1st week after surgery, the LAD lumens were narrowed 84% ± 5% (mean ± SE) by neointimal proliferation and/or organizing thrombi. In 2 dogs with mild to moderate CFVs, defined as more than 3 flow reductions that were 30–60% of baseline flow velocity levels, neointimal proliferation resulted in 40% ± 5% narrowing of the LAD lumen. In the remaining animals without CFVs, only 17% ± 4% narrowing of the LAD lumen by neointimal proliferation was observed. The correlation between the severity and frequency of CFVs and the severity of neointimal proliferation was significant (Pearson's $r = 0.90$; $P \leq 0.001$) (Fig. 3).

Prevention of Neointimal Proliferation. Treatment with the combined thromboxane synthetase inhibitor and receptor antagonist (ridogrel) and the serotonin receptor antagonist (ketanserin or LY53857) was started during surgery immediately after the appearance of the first one or two CFVs, and they initially eliminated CFVs within 5–10 min in all animals. *Ex vivo* platelet aggregations induced by U46619 and serotonin were also inhibited. After surgery, 7 dogs received injections of the two agents every 8 hr for 7–14 days. CFVs still developed in 4 of the 7 dogs under this regimen. The addition of continuous infusions of the two agents to the injections at 8-hr intervals prevented CFVs in 10 additional dogs. Coronary artery neointimal proliferation in the 4 dogs in whom CFVs were not prevented was similar to that found in untreated animals. In the dogs in whom ridogrel and ketanserin were effective in preventing CFVs, there was less neointimal proliferation (14% ± 6%) (Figs. 4 and 5). Marked inhibition of the neointimal proliferation was observed in 8 of 10 dogs treated with bolus injections at 8-hr intervals and continuous infusions of ridogrel and ketanserin in whom *ex vivo* platelet aggregations induced by the thromboxane mimetic, U46619, and serotonin were also inhibited during the entire period of treatment (Figs. 4 and 5). These data suggest that marked attenuation or inhibition of recurrent platelet aggregation at sites of endothelial injury and coronary artery stenosis may be necessary to markedly attenuate coronary artery neointimal proliferation in this experimental model. The data also demonstrate the "malignant" nature of platelet aggregation at sites of endothelial injury and coronary stenosis in this experimental model and the relative difficulty in preventing its occurrence, thereby leading to the requirement for continuous infusions of a combined thromboxane synthesis inhibitor and receptor antagonist and a serotonin receptor antagonist for protection. Two dogs developed mild to moderate neointimal proliferation (31% and 42% luminal diameter narrowings) after continuous infusions of ridogrel and ketanserin and prevention of CFVs as well as inhibition of *ex vivo* platelet aggregation (Fig. 5). This suggests that factors in addition to recurrent platelet aggregation may contribute to the development of neointimal proliferation. Alternatively, these two animals may have had enough platelet deposition and growth factor activation to initiate moderate neointimal proliferation even though recurrent platelet attachment and dislodgement in the form of CFVs could not be identified.

DISCUSSION

The processes that lead to neointimal proliferation after endothelial injury are incompletely understood. Platelet-

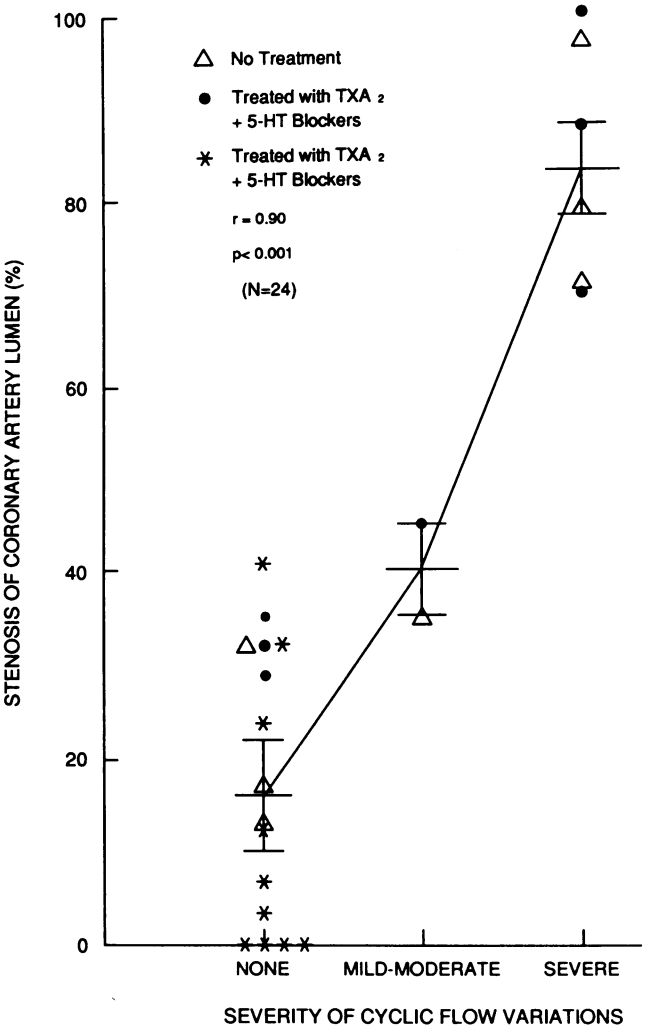

FIG. 4. Correlation between the frequency and severity of coronary CFVs and the severity of coronary artery neointimal proliferation is shown. ●, Dogs received ridogrel [a dual thromboxane A_2 (TXA_2) synthetase and receptor antagonist] at 5 mg/kg and ketanserin [a serotonin (5-HT) receptor antagonist] at 0.5 mg/kg or LY53857 (another serotonin receptor antagonist) at 0.2 mg/kg in bolus doses every 8 hr for 7–14 days. *, Dogs received ridogrel 5–10 mg/kg every 8 hr plus continuous infusions of 0.6 $mg \cdot kg^{-1} \cdot hr^{-1}$ and ketanserin at 1–2 mg/kg every 8 hr plus continuous infusions of 0.1–0.2 $mg \cdot kg^{-1} \cdot hr^{-1}$ for 14 days.

derived growth factors (PDGFs) as well as several other mitogens induce smooth muscle cell migration and proliferation (30–37). PDGFs are released from α granules of platelets after their activation and adhesion. Inhibition of platelet aggregation would therefore be a logical step in attempting to prevent neointimal proliferation. Of course, growth factors may be released from other cells and tissues as well, including mononuclear cells and endothelium. Clinical use of antiplatelet agents, such as aspirin, has not prevented neointimal proliferation leading to restenosis after coronary angioplasty in patients with coronary artery disease (38). Data from the present study stress the relatively malignant nature of recurrent platelet aggregation and dislodgement following mechanically induced endothelial injury and the need to give sustained antiplatelet therapy with combined inhibitors for thromboxane A_2 and serotonin in this experimental model. Therefore, insufficient inhibition of recurrent platelet aggregation when just aspirin is used might be at least one factor leading to the inability to markedly attenuate the neointimal proliferation observed in previous studies.

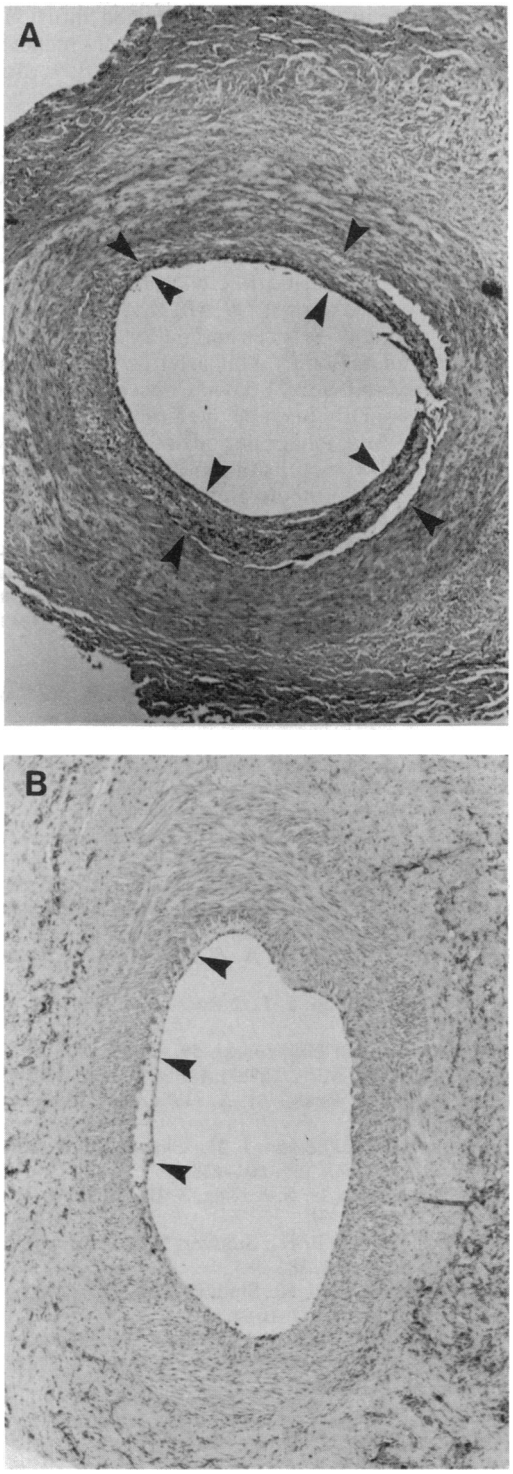

FIG. 5. Micrographs of the LAD from dogs 21 days after endothelial injury with their coronary CFVs prevented by treatment with ridogrel and ketanserin are shown. (*A*) Dog that received ridogrel at 5 mg/kg and ketanserin at 0.5 mg/kg every 8 hr for 14 days. A moderate arterial lumen stenosis caused by neointimal proliferation (tissue between arrowheads) was observed. (Hematoxylin and eosin; ×60.) (*B*) Dog that received ridogrel at 5 mg/kg every 8 hr with a continuous infusion of 0.6 $mg \cdot kg^{-1} \cdot hr^{-1}$ and ketanserin at 1 mg/kg every 8 hr with a continuous infusion of 0.1 $mg \cdot kg^{-1} \cdot hr^{-1}$ for 2 weeks. There was minimal neointimal proliferation (arrowheads) within the constricted and endothelium-injured site. (Hematoxylin and eosin; ×45.)

Recurrent platelet aggregation appears to play an important role in development of coronary arterial neointimal proliferation after endothelial injury in this experimental

model. Bolus administration and sustained infusions of a thromboxane synthesis inhibitor and receptor antagonist and a serotonin receptor antagonist prevent cyclic flow variations and usually markedly attenuate neointimal proliferation that occurs at sites of endothelial injury in this experimental model. However, complete prevention of neointimal proliferation may require large doses of specific inhibitors of platelet mediators, other antiplatelet strategies, direct inhibition of smooth muscle cell migration and/or proliferation, and/or antagonists of growth factor receptors or postreceptor events to block several critical activation pathways simultaneously. Others have shown (39, 40) that profound thrombocytopenia markedly reduces intimal proliferation in mechanically injured rat and rabbit arteries. In addition, our observations are limited to 21 days. Conceivably, neointimal proliferation has simply been delayed rather than prevented by this therapy. Thus, longer periods of experimental observation to include 3–6 months of follow-up will be necessary to prove a persistent protective effect.

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