Inhibitory Effect of Clopidogrel on Platelet Adhesion and Intimal Proliferation After Arterial Injury in Rabbits

J.M. Herbert, A. Tissinier, G. Defreyn, and J.P. Maffrand

The possible activity of ticlopidine and its analogue clopidogrel in early atherogenesis was investigated. Incubation of rabbit platelets with the extracellular matrix produced by endothelial cells in culture induced massive platelet adherence in vitro. This phenomenon was strongly reduced when platelets were isolated from rabbits that had been treated with a single dose of clopidogrel (10 mg/kg PO) or three doses of ticlopidine (each 200 mg/kg PO) (94% and 56% inhibition of platelet adhesion, respectively). Three doses of aspirin (each 200 mg/kg PO) were ineffective. Air-drying injury of the rabbit carotid artery resulted in platelet adherence to the underlying subendothelium. This platelet accumulation on the damaged vessel wall was greatly reduced by clopidogrel; 95% (P<.001) inhibition of platelet adhesion after a single oral dose of 25 mg/kg. Ticlopidine (200 mg/kg) was also effective (71% inhibition; P<.001), whereas aspirin (100 mg/kg PO) failed to reduce platelet adhesion to the subendothelium. The effect of clopidogrel on intimal smooth muscle hyperplasia in rabbit carotid arteries subjected to air-drying endothelial injury was then investigated. After a 16-day treatment, clopidogrel (25 mg/kg per day PO) inhibited the development of intimal thickening (48% inhibition; P<.01). This effect was dose dependent and increased with the duration of the treatment. Under the same experimental conditions, ticlopidine (200 mg/kg per day PO) inhibited myointimal thickening (57%; P<.001), whereas aspirin was ineffective. These results show that clopidogrel and ticlopidine, two ADP-selective antiplatelet agents, can reduce myointimal thickening after endothelial injury. This effect can be due to the inhibition of platelet adhesion and aggregation to the exposed subendothelium. (Arteriosclerosis and Thrombosis 1993;13:1171-1179)

KEY WORDS • clopidogrel • platelets • adhesion • smooth muscle cells • proliferation • injury • rabbits

The phenomenon of restenosis, which can occur after mechanical dilatation of a coronary stenosis by means of a percutaneous transluminal coronary angioplasty (PTCA), needs to be studied to determine the clinical success of this intervention therapy in ischemic heart disease. To aid in the prevention of restenosis occurring after PTCA, a number of antiplatelet drugs such as dipyridamole/aspirin, aspirin alone, or ticlopidine have been widely administered during or shortly after PTCA. Until now, none of these compounds have reduced the restenosis that occurs in 30% to 40% of the PTCA procedures performed in humans.

The process of intimal thickening in an injured artery is the consequence of smooth muscle cell (SMC) proliferation and migration from the media to the intima. The specific role of platelets in this cellular proliferation has been suggested by work on drugs affecting platelet aggregation, although the general contribution of platelets and thrombosis to the development of atherosclerotic plaques has been acknowledged for a long time.

Previous investigations suggested that platelet adherence to the thrombogenic subendothelium, followed by extensive aggregation and secretion of platelet factors, may provide the mitogenic stimulus for dormant SMCs in the arterial wall, and it has been recently suggested that platelets regulate the movement of SMCs into the intima but that they do not play a role in the initiation of proliferation.

The hypothesis that intimal hyperplasia results from vessel injury and platelet responses to the exposed subendothelium is supported by experiments in animals. Rabbits on a normal diet developed fibromuscular plaques in response to de-endothelialization with a balloon catheter. This was completely inhibited if the animals were made severely thrombocytopenic with antiplatelet antiserum. SMC proliferation induced in animals by endothelial injury was reported to be inhibited by treatment with antiaggregating drugs such as a thiazole compound or ticlopidine. Antiplatelet therapy with a combination of dipyridamole and aspirin has also been effective in reducing intimal hyperplasia in response to coronary artery and femoralpliteal bypass grafts in dogs and nonhuman primates and in slowing the progression of peripheral arterial disease. These observations are therefore inconsistent with the effect of these same compounds on restenosis occurring after PTCA in humans. There may be a number of explanations for the difference between these re-
sponses, a possible explanation being the low antiadhesive effect of all of these compounds in humans. Indeed, this latter effect appears to be of particular importance in the initiation of restenosis, since another model frequently cited to support platelet involvement in atherosclerosis is the swine homozygous for von Willebrand's disease. These pigs have prolonged bleeding times and a deficiency of the von Willebrand factor necessary for normal platelet adherence to the subendothelial tissue. These swine are less susceptible to aortic atherosclerosis either induced by atherogenic diet alone or associated with balloon endothelial injury.

The close interactions between platelets and the response to vascular injury suggest that drugs that strongly inhibit platelet functions, such as adhesion and/or aggregation, should reduce the extent of lesion development after injury. The aim of this study was to examine the effect of clopidogrel, a ticlopidine analogue and a powerful inhibitor of ADP-induced platelet aggregation, on platelet adherence to the subendothelium in vitro and in vivo and to evaluate its activity on myointimal proliferation after air-drying injury of the rabbit carotid artery.

Methods

In Vitro Experiments

Corneal endothelial cell cultures and preparation of the extracellular matrix (ECM). Cultures of bovine corneal endothelial cells were established from steer eyes as described. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum, 5% fetal calf serum (FCS), 4 mmol/L glutamine, 100 μg/mL streptomycin sulfate, and 100 IU/mL penicillin at 37°C in 10% CO₂ humidified incubators. Cells were passaged weekly at a split ratio of 1:4, and 1 ng/mL basic fibroblast growth factor (Amersham, France) was added every other day. Corneal endothelial cells at the third passage were plated at an initial density of 1.8x10⁶ cells per well in 24-well cluster plates (Nunc, Roskilde, Denmark) and cultured under the same conditions as described above except that 5% dextran T-40 (Sigma) was added in the growth medium. Eight days after confluency was reached, the cell layer was washed twice with phosphate-buffered saline (PBS) and exposed to 0.5% Triton X-100 in PBS (vol/vol) for 30 minutes under gentle shaking. The remaining cytoskeleton and nuclei were removed by a 2-minute exposure to 0.025 mol/L NH₄OH (1 mL/well), and the radioactivity of the lysate was measured in a gamma counter (model MR 480, Kontron).

Rabbit aortic SMC culture in vitro. SMCs were isolated from the rabbit aorta as described previously. Briefly, media fragments of the thoracic aorta from New Zealand White rabbits were incubated for 16 hours at 37°C in DMEM containing 0.15% collagenase, 5% FCS, penicillin (100 IU/mL), streptomycin (100 μg/mL), and glutamine (4 mmol/L) (Boehringer Mannheim). After incubation, SMCs were sedimented by gentle centrifugation (400g for 10 minutes), resuspended in DMEM+10% FCS, and grown at 37°C in a humidified atmosphere of 5% CO₂ in air. Culture medium (DMEM+10% FCS) was changed every 3 days and a confluent SMC monolayer was obtained after about 7 days. Cells were routinely used from the third to the sixth passage. To determine the effect of the various compounds on their proliferative response, cells were plated sparsely (10⁴ cells per well) in 96-well cluster plates (Nunc) in DMEM+0.5% FCS. After 3 days, cells in representative dishes were counted with a Coulter counter (Coultronics), and fresh medium was added to the remaining dishes (DMEM+5% FCS and the different concentrations of the drugs to be tested). For growth rate determinations, after 3 days in culture cells were detached from triplicate wells by trypsin treatment (0.05% trypsin/0.02% EDTA) and counted in a Coulter counter. Median inhibitory concentration (IC₅₀) values were calculated on the basis of the linear regression lines established for each compound tested by using a regression program.

In Vivo Experiments

Air-drying injury. Male New Zealand White rabbits (Charles River) weighing 2.5 to 3 kg were used. Air-drying injury was induced by applying an air flow through the carotid artery using a modification of Fishman's method. Briefly, rabbits were anesthetized with sodium pentobarbital (30 mg/kg IV), and the left carotid artery was exposed and ligated at two points 1.5 cm apart. A 27-gauge hypodermic needle was inserted through the segment at 240 mL/min for 5 minutes. Ligatures were then removed, allowing circulation to be reestablished, hemostasis was ensured, and the incision was closed.

Tissue preparation and morphological examination. The antiplatelet drugs were administered orally 2 hours before carotid injury except for window experiments, in which clopidogrel was first administered 24 hours after air-drying injury. Sixteen days after surgery, animals were anesthetized with sodium pentobarbital (30 μg/mL) and a stream of dry air was allowed to flow through the segment at 240 mL/min for 5 minutes. Ligatures were then removed, allowing circulation to be reestablished, hemostasis was ensured, and the incision was closed.

Downloaded from http://atvb.ahajournals.org/ by guest on October 30, 2017
Drugs

Ticlopidine (Ticlid), clopidogrel [p-methyl(2-chlorophenyl)-5-(4,5,6,7-tetrahydrothieno)(3,2-c)pyridinyl]acetate, hydrogen sulfate] and the corresponding inactive L-enantiomer (SR 25989) were from Sanofi Recherche (Toulouse, France). Aspirin was purchased from Sigma. All compounds were administered orally in a 5% gum arabic solution in water.

Statistical Analysis

All data are expressed as mean±1 SD. The n values indicate the number of animals studied. Grouped data were analyzed for significance by comparison with the vehicle-treated group by using the Kruskal-Wallis or Mann-Whitney U test as indicated. The level of significance was chosen as P<.05.

Results

Effect of Clopidogrel on Platelet Adhesion to the ECM

When 111In-labeled rabbit platelets were added to uncoated plastic dishes, little attachment occurred (Fig 1). In contrast, the addition of increasing concentrations of platelets from untreated rabbits to the ECM produced by bovine corneal endothelial cells resulted in rapid and massive adherence to the ECM. Platelets spread on the ECM, developed pseudopods, and formed a monolayer as visualized by phase-contrast microscopy. Under these experimental conditions, large aggregates composed of several layers of platelets were never found.

Treatment of the animals with a single oral dose of clopidogrel (10 mg/kg PO) 2 hours before platelet preparation abolished platelet reactivity, as revealed by almost complete inhibition of platelet adhesion (94%; P<.001) (Table 1, Fig 1). Under these conditions, ADP-induced platelet aggregation was reduced by 86%. The L-enantiomer of clopidogrel, SR 25989, known to be inactive on ADP-induced platelet aggregation,29 did not have any effect on platelet adherence to the ECM.

FIG 1. Line graph showing ex vivo effect of clopidogrel on platelet adhesion to the extracellular matrix (ECM). Increasing concentrations of 111In-labeled platelets prepared from rabbits treated 2 hours before being administered single oral doses of clopidogrel (10 mg/kg; •); SR 25989 (50 mg/kg; ○); or the vehicle (□) were incubated with the ECM for 60 minutes at 25°C. Platelets from the vehicle-treated rabbits were also incubated under the same experimental conditions without the ECM (○). The amount of ECM-bound platelets was determined as described in “Methods.” Results are expressed as mean±SD; n=3.

mg/kg IV), and 200 IU/kg heparin (Sigma) was injected. The aorta was then perfused with saline followed by 4% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. Both common carotid arteries were excised and fixed by immersion for an additional 4 hours in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. The vessels were washed in buffer, postfixed for 2 hours in 2% aqueous OsO4, and stained in 2% aqueous uracil acetate en bloc. They were then dehydrated through graded solutions of alcohol and embedded in Epon 812 for serial cross sectioning. Epon sections (2 μm) were stained with toluidine blue. Morphometric analysis of arterial sections was done by use of the Biocom Imagenia 2000 image analysis system (Biocom, Lyon, France). Plaque size was determined for each segment by tracing the luminal perimeter and internal and external elastic laminae of each section with a digitizing tablet. For intimal and medial thicknesses, the average of five equally spaced radial measurements for each section was used.

Determination of platelet deposition on the subendothelium. To quantify platelet adherence to the injured arterial wall, the rabbits were injected with homologous 111In-platelets 1 hour before removing the injured vessel wall segment (1 to 24 hours after air injury). Immediately before removal of the segment, a 5-mL citrated blood sample was collected from the rabbit for the determination of whole-blood platelet-specific activity. Each rabbit was then heparinized (250 IU/kg) to prevent thrombin generation and postmortem platelet accumulation and killed 2 minutes later with an overdose of sodium pentobarbital. A standard length (1.5 cm) of the abdominal aorta at 120 mm Hg pressure with 1% paraformaldehyde. A standard length (1.5 cm) of each vessel was removed and put in a liquid scintillation vial containing 1 mL of 1N NaOH. One hour later, the vessel was rinsed with 10 mL of toluene-based counting fluid, and the number of adherent platelets per square micrometer sputter-coating device. The specimens were examined by scanning electron microscopy at 1, 4, and 24 hours after air-drying injury. Animals were anesthetized with sodium pentobarbitone (30 mg/kg IV) and received an intravenous injection of heparin (200 IU/kg). The abdomen was opened and a retrograde cannula was inserted into the abdominal aorta. Carotid arteries were rinsed with 10 mL of toluene-based counting fluid, and the number of adherent platelets per square micrometer. The specimens were examined by scanning electron microscopy

Scanning electron microscopy. In another group of animals, the inner surface of the left carotid artery was studied by scanning electron microscopy at 1, 4, and 24 hours after air-drying injury. Animals were anesthetized with sodium pentobarbitone (30 mg/kg IV) and received an intravenous injection of heparin (200 IU/kg). The abdomen was opened and a retrograde cannula was inserted into the abdominal aorta. Carotid arteries were fixed by retrograde perfusion via the abdominal aorta at 120 mm Hg pressure with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. Excised tissues, including right and left common carotid arteries, were further fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde for 2 to 4 hours. Arteries were postfixed in 2% OsO4 and dehydrated using alcohols. They were sectioned at the midpoint, cut longitudinally, critical-point dried in CO2 and coated with gold/palladium with a Technics Hummer sputter-coating device. The specimens were examined at 30 kV with a Philips SEM 505 scanning electron microscope.

Morphometric analysis of arterial sections was done by use of the Biocom Imagenia 2000 image analysis system (Biocom, Lyon, France). Plaque size was determined for each segment by tracing the luminal perimeter and internal and external elastic laminae of each section with a digitizing tablet. For intimal and medial thicknesses, the average of five equally spaced radial measurements for each section was used.

Effect of Clopidogrel on Platelet Adhesion to the ECM

When 111In-labeled rabbit platelets were added to uncoated plastic dishes, little attachment occurred (Fig 1). In contrast, the addition of increasing concentrations of platelets from untreated rabbits to the ECM produced by bovine corneal endothelial cells resulted in rapid and massive adherence to the ECM. Platelets spread on the ECM, developed pseudopods, and formed a monolayer as visualized by phase-contrast microscopy. Under these experimental conditions, large aggregates composed of several layers of platelets were never found.

Treatment of the animals with a single oral dose of clopidogrel (10 mg/kg PO) 2 hours before platelet preparation abolished platelet reactivity, as revealed by almost complete inhibition of platelet adhesion (94%; P<.001) (Table 1, Fig 1). Under these conditions, ADP-induced platelet aggregation was reduced by 86%. The L-enantiomer of clopidogrel, SR 25989, known to be inactive on ADP-induced platelet aggregation,29 did not have any effect on platelet adherence to the ECM.

Determination of platelet deposition on the subendothelium. To quantify platelet adherence to the injured arterial wall, the rabbits were injected with homologous 111In-platelets 1 hour before removing the injured vessel wall segment (1 to 24 hours after air injury). Immediately before removal of the segment, a 5-mL citrated blood sample was collected from the rabbit for the determination of whole-blood platelet-specific activity. Each rabbit was then heparinized (250 IU/kg) to prevent thrombin generation and postmortem platelet accumulation and killed 2 minutes later with an overdose of sodium pentobarbital. A standard length (1.5 cm) of the abdominal aorta at 120 mm Hg pressure with 1% paraformaldehyde. A standard length (1.5 cm) of each vessel was removed and put in a liquid scintillation vial containing 1 mL of 1N NaOH. One hour later, the vessel was rinsed with 10 mL of toluene-based counting fluid, and the number of adherent platelets per square micrometer. The specimens were examined by scanning electron microscopy

Scanning electron microscopy. In another group of animals, the inner surface of the left carotid artery was studied by scanning electron microscopy at 1, 4, and 24 hours after air-drying injury. Animals were anesthetized with sodium pentobarbitone (30 mg/kg IV) and received an intravenous injection of heparin (200 IU/kg). The abdomen was opened and a retrograde cannula was inserted into the abdominal aorta. Carotid arteries were fixed by retrograde perfusion via the abdominal aorta at 120 mm Hg pressure with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. Excised tissues, including right and left common carotid arteries, were further fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde for 2 to 4 hours. Arteries were postfixed in 2% OsO4 and dehydrated using alcohols. They were sectioned at the midpoint, cut longitudinally, critical-point dried in CO2, and coated with gold/palladium with a Technics Hummer sputter-coating device. The specimens were examined at 30 kV with a Philips SEM 505 scanning electron microscope.
Effect of Clopidogrel on Rabbit SMC Growth

After 3 days in culture, triplicate wells were trypsinized (•). Cells were counted. Data are expressed as mean percent inhibition of proliferation compared with replicate cultures grown without the inhibitor (n=3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Inhibition (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>200†</td>
<td>4±2</td>
<td>NS</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>100†</td>
<td>21±5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>200†</td>
<td>56±9</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>1</td>
<td>12±4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>41±7</td>
<td>.01</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>94±5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SR 25989</td>
<td>50</td>
<td>2±1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Inhibition data are expressed as mean±SD. NS, not significant.
†Compounds were administered to the animals for 3 days before the experiment.

Under the same experimental conditions, aspirin administered for 3 days at a daily oral dose of 200 mg/kg induced no activity. Ticlopidine at the same dosage strongly affected platelet adhesion to the ECM (56%; P<.001) (Table 1).

When clopidogrel was incubated in vitro (10 μmol/L) for 1 hour in the presence of the ECM and platelets from untreated animals, no effect on platelet adhesion occurred, therefore confirming that the in vivo metabolism is necessary for the antiplatelet effect of this class of compounds. Similarly, ticlopidine failed to reduce platelet adhesion when the ECM was obtained from endothelial cells grown for 16 days in the presence of 150 μmol/L ticlopidine.

Effect of Clopidogrel on Rabbit SMC Growth

As shown in Fig 2, neither clopidogrel nor ticlopidine up to a concentration of 400 μmol/L altered serum-induced rabbit aortic SMC growth. When used as a control, standard heparin reduced SMC proliferation with an IC\(_50\) value of 250 μg/mL. This result is consistent with already published observations.\(^{26,30,31}\)

Effect of Clopidogrel on Platelet Adhesion to the Air-Injured Rabbit Carotid Artery

Scanning electron photomicrographs of the luminal surface of an uninjured carotid artery are shown in Fig 3, A. One hour after air dessication, both spread and discoid platelets were found in close contact with the remaining endothelial surface (Fig 3, B). These cells were mainly in the margins or between the endothelial cells, and traces of fibrin were present. This phenomenon becomes more evident in Fig 3, C, which shows the morphology of the luminal surface 4 hours after air-drying injury. At this time point, all the endothelial cells had been removed from the full circumference of the segment of the artery that had been exposed to dessication, and the luminal surface consisted of a monolayer of spread platelets with some small aggregates and fibrin. There were also large numbers of polymorphonuclear leukocytes (up to approximately 1000/mm\(^2\)) associated with the platelet layer. Twenty-four hours after air drying, the injured surface was composed of irregularly shaped red blood cells linked with moderate to extensive amounts of fibrin and large numbers of aggregated platelets (Fig 3, D).

Parallel quantification of \(^{11}\)In-labeled platelet adherence to the air-injured artery indicates that after an initial rise, which occurred within the first 4 hours, the number of bound \(^{11}\)In-platelets reached a plateau 24 hours after de-endothelialization (Fig 4). At 24 hours, the injured carotid artery of aspirin-treated animals showed \(^{11}\)In-labeled platelet adherence of the same magnitude as that observed in untreated animals (Fig 3, E, Table 2). However, a single oral administration of clopidogrel (25 mg/kg) resulted in strong inhibition of platelet adherence to the subendothelium (Fig 4, Table 2, and Fig 3, F) 24 hours after the air-drying injury. A similar effect was obtained with daily doses of ticlopidine (200 mg/kg PO) for 6 days.

Effect of Clopidogrel on Myointimal Proliferation After Air-Drying Injury

In the right carotid artery (which had not been subjected to air injury) there was no evidence of intimal proliferation, foam cell formation, or platelet accumulation 16 days after the surgical procedure, indicating that physical manipulation at the time of surgery without air-drying injury was not sufficient to generate the lesion. However, the time course of proliferation in the intimal region of the dessicated left rabbit carotid artery indicated that after denudation SMCs can be observed in the intima by day 7 (Fig 5). By 2 weeks the intima had grown substantially, and it stayed at the same size for the remainder of the experimental period. Sixteen days after endothelial denudation, the intimal surface represented 35±7% of the tunica media area. Morphometric analysis revealed that the changes in the increase in the intimal diameter occurred while the medial diameter remained mostly unchanged (Fig 5). This neointima, composed of up to eight layers of SMCs, extended to the entire circumference and approximately 80% of the length of the injured area. The SMCs of the neointima
Fig 3. Scanning electron photomicrographs (SEMs) of the luminal surface of an injured control rabbit carotid artery before (A) and at 1 hour (B), 4 hours (C), and 24 hours (D) after injury. SEMs of the arterial luminal surface of the carotid artery isolated 24 hours after injury from rabbits treated with aspirin (100 mg/kg PO) (E) or clopidogrel (25 mg/kg PO) (F). Bar=10 μm.
and the superficial media were mainly of the secretory phenotype, as described by Richardson et al.\(^*\)

As shown in Fig 5 and Table 3, a 16-day oral treatment of clopidogrel (25 mg/kg per day) strongly reduced SMC hyperplasia after de-endothelialization (48% inhibition; \(P<.01\)). A substantial increase in this effect was observed when the animals were treated for a 23-day period (61% inhibition; \(P<.001\)) (Fig 5). This effect was dose related, because an oral dose of clopidogrel (10 mg/kg per day) resulted in 21% inhibition of neointimal formation (\(P<.05\)). To determine what part inhibition of platelet adhesion plays in the subsequent effect of clopidogrel on myointimal proliferation, we performed window experiments in which clopidogrel treatment was started 24 hours after de-endothelialization. A 24-hour delay in clopidogrel administration resulted in a dramatic reduction of its efficacy on either platelet adhesion or myointimal proliferation (Table 4). Under treatment with clopidogrel (25 mg/kg per day PO), the morphology of the SMCs near the medial surface varied in comparison with the SMCs in the remaining neointima. Indeed, these SMCs showed decreased amounts of metabolic organelles compared with SMCs of the neointima from controls. After 23 days, the neointima of the treated animals was two to three layers thick, with SMCs showing prominent myofilaments (Fig 5). This morphology is a typical feature of SMCs in a contractile phenotype.\(^2\) Reduction in intimal thickening was also observed after a 16-day administration of ticlopidine (200 mg/kg per day PO) (57% inhibition; \(P<.001\)) (Table 3), whereas aspirin did not significantly affect myointimal proliferation after air injury. These observations have already been shown elsewhere.\(^13\)\(^3\) It is noteworthy that none of these compounds affected re-endothelialization of the denuded area.

### Discussion

Restenosis after successful PTCA remains one of the major unresolved problems with this procedure. Restenosis occurs in 30% to 40% of the dilated lesions, usually 3 to 4 months after PTCA.\(^1\)\(^3\) Although restenosis can be effectively treated by repeating the PTCA procedure, the large number of patients requiring second and even third operations adds significantly to morbidity and cost, and despite numerous clinical trials and animal studies, no other nonmechanical, effective treatment has been found. Several mechanisms were initially proposed as possible causes of restenosis, including organized thrombus

### Table 2. Effects of Aspirin, Ticlopidine, and Clopidogrel on Platelet Adhesion to the Rabbit Carotid Artery After De-endothelialization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg per day)</th>
<th>Inhibition (%)</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>100</td>
<td>4±1</td>
<td>NS</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>200†</td>
<td>63±9</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>25</td>
<td>93±7</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Inhibition data are expressed as mean±SD. NS, not significant.

\( *\)Mann-Whitney U test; \( n = 7 \) to 11.

†Compound was administered to the animals for 6 days before the experiment.

### Table 3. Effect of Aspirin, Ticlopidine, and Clopidogrel on the Myointimal Proliferation of the Rabbit Carotid Artery After De-endothelialization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg per day)</th>
<th>Inhibition (%)</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>100</td>
<td>17±6</td>
<td>NS</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>200</td>
<td>57±11</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>25</td>
<td>48±9</td>
<td>&lt;.01</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21±4</td>
<td>.05</td>
</tr>
</tbody>
</table>

Inhibition data are expressed as mean±SD. NS, not significant.

\( *\)Kruskal-Wallis test; \( n = 9 \) to 15.
subendothelium result in the release of growth factors such as platelet-derived growth factor and epidermal growth factor and other humoral factors such as serotonin, histamine, and norepinephrine, which are mitogenic and/or chemotactic for SMCs. Other factors, such as shear stress or the infiltration of inflammatory cells at the injured site, might also influence the final outcome of intimal hyperplasia. Although a large number of factors may contribute to this process, platelets appear to play a fundamental role in initiating and maintaining intimal hyperplasia. Therefore, one possible method for reducing SMC proliferation after arterial injury might be to administer drugs that inhibit platelet adhesion and/or aggregation to collagen or other reactive components of the arterial wall.

In this study, we attempted to associate the in vitro adherence of platelets from rabbits treated with aspirin, ticlopidine, or clopidogrel to the in vivo deposition of similar platelets on the exposed carotid artery subendothelial layers. We further extended our investigations to determine the effect of these same compounds on myointimal proliferation after air-drying injury of the vessel wall. Our results demonstrate that, although used at doses that have been widely described to markedly reduce platelet functions, aspirin did not alter the in vitro pattern of platelet interactions with the ECM produced by endothelial cells in culture. Similar results have been reported in other models. However, ticlopidine and its potent analogue clopidogrel strongly counteracted platelet adhesion to the subendothelium. This effect was dose dependent for both compounds and was observed at concentrations that have been shown to reduce ADP-induced platelet aggregation ex vivo. As already shown for platelet aggregation, the L-antagonist of clopidogrel (SR 25989) did not significantly affect platelet adhesion to the ECM, thus demonstrating the specificity of the observed effect.

Such observations can be compared with results showing that ticlopidine inhibits platelet adhesion to foreign surfaces such as glass beads, Gore-Tex, or Dacron grafts in dogs. Unlike what was observed for aspirin, ticlopidine and clopidogrel are both potent inhibitors of platelet adhesion in vitro, but in spite of the high degree of similarity between the ECM produced by endothelial cells in culture and the basal lamina of the vascular endothelium, the possible role that vascular prostaglandin I2 and rheological parameters could play in such a phenomenon was disregarded. Thus, the adherence of platelets to the subendothelium was studied in vivo after exposure of the subendothelium after injury of the rabbit carotid artery.

Platelet accumulation was examined after extensive endothelial removal by air-drying injury. Our observations confirmed other work showing that platelet interactions with the exposed subendothelium were minimal during the first 4 hours after endothelial desquamation. The denuded surface was then rapidly covered by platelets, which spread and discharged a variable amount of their granule content. In the present study, although there was minimal medial damage, neutrophils accumulated on the arterial surface at early time points and infiltrated the vessel wall. This neutrophil infiltration and associated edema of the media that occurred shortly after injury are typical of an inflammatory response and are consistent with other observations.

or vasospasm, but a number of autopsy studies have shown that intimal hyperplasia is the main histological finding of restenotic coronary arteries in patients with prior successful PTCA. During vessel damage, such as balloon injury, the release and production of growth-stimulatory and chemotactic factors occurs, as does the alteration and destruction of the spatial arrangements of SMCs and the ECM. SMCs start to proliferate as a result of exposure to growth factors and the loss of endothelial inhibitory influences. Platelet adhesion and aggregation caused by exposure of the thrombogenic

<table>
<thead>
<tr>
<th>Clopidogrel (25 mg/kg per day)</th>
<th>Inhibition of platelet adhesion (%)</th>
<th>Inhibition of intimal thickening (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Administered 2 h before injury and daily for 16 d</td>
<td>93±7</td>
<td>48±9</td>
</tr>
<tr>
<td>Administered 24 h after injury and daily for 16 d</td>
<td>7±3</td>
<td>4±1</td>
</tr>
</tbody>
</table>

Fig 6. Scanning electron photomicrographs showing effect of clopidogrel on the phenotype of intimal smooth muscle cells (SMCs) of the rabbit carotid artery. Vehicle (A) or clopidogrel (25 mg/kg PO; B) were administered 2 hours before air injury and daily for 16 days to rabbits whose carotid artery was de-endothelialized. A, SMCs present in the neointima of control animals. Cells are in a synthetic state and their cytoplasm is filled with large amounts of free ribosomes, rough endoplasmic reticulum, and mitochondria. B, SMCs present in the neointima of clopidogrel-treated animals. Thick and thin myofilaments in the cytoplasm are seen in SMCs in a contractile phenotype. Bar=2 μm.
As already reported, aspirin remained without effect on platelet deposition on the arterial wall denuded of endothelium, whereas platelet interactions with the subendothelial connective tissue can be almost totally prevented by a single dose of clopidogrel (25 mg/kg PO) or repeated daily doses of ticlopidine (200 mg/kg PO) for 6 days. These results therefore confirm those obtained on platelet adhesion to the ECM in vitro and in vivo but conflict with data from Cattaneo et al., who showed that ticlopidine did not affect platelet adhesion in other animal models, such as balloon-deendothelialized rabbit aortas or platelet accumulation induced by an indwelling catheter in rat carotid arteries. Nevertheless, although abundant evidence of the effectiveness of antiplatelet drugs in preventing platelet aggregation is now available, studies on the influence of such drugs on the in vivo deposition of platelets on injured vessels have produced contradictory results and seem to vary with the experimental model.

To our knowledge, however, clopidogrel is considered to be one of the most potent compounds inhibiting platelet adherence to the subendothelium in vivo.

Since it has been demonstrated that these compounds interfere directly with platelet adhesion, an important initial step in restenosis, we decided to determine their effect on arterial SMC proliferation (intimal hyperplasia) in vivo. As a result of air drying, intimal thickening reached a maximum of eight SMC concentric layers by 2 weeks and showed no further increase by 2 months (not shown). After 2 weeks, changes in the phenotype were also observed for SMCs in the neointima, which were mainly of the secretory phenotype (Fig 6).

In this model, aspirin showed no effect on the morphology of the lesion or on its development, therefore confirming data reported previously by Clowes and Karnovsky. Arterial hyperplasia was, however, significantly reduced by a daily dose of ticlopidine (200 mg/kg PO). Ticlopidine, at a dose similar to that used in our study, has been reported to inhibit intimal SMC proliferation by balloon catheter endarterectomy in the rabbit abdominal aorta. Under the same experimental conditions, clopidogrel exhibited dose-dependent inhibition of intimal thickening. As already shown for ADP-induced platelet aggregation, not only was clopidogrel interrupted de-endothelialization. On this basis, one could assume that compounds related to ticlopidine, such as clopidogrel, with greater potency as ADP-selective inhibitors, may be useful in preventing arterial hyperplasia in humans.

Acknowledgment

The authors thank A.J. Patacchini for her helpful comments on the manuscript.

References

15. Donaldson DR, Saltz NC, Kester RC, Rajah SM, Hall TJ, Sreeharan N, Crow MJ. The influence of platelet inhibition on the
Inhibitory effect of clopidogrel on platelet adhesion and intimal proliferation after arterial injury in rabbits.

J M Herbert, A Tissinier, G Defreyn and J P Maffrand

doi: 10.1161/01.ATV.13.8.1171

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/8/1171

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/