Journal of the American College of Cardiology Vol. 46, No. 6, 2005

ISSN 0735-1097/05/$30.00
doi:10.1016/j.jacc.2005.02.094

FOCUS ISSUE: ASPIRIN

Aspirin, But Not Clopidogrel, Reduces Collateral Conductance in a Rabbit Model of Femoral Artery Occlusion

Imo E. Hoefer, MD, PHD,*†‡ Sebastian Grundmann, MD,*† Stephan Schirmer, MD,*† Niels van Royen, MD, PHD,§ Benjamin Meder, MD,* Christoph Bode, MD,* Jan J. Piek, MD, PHD,§ Ivo R. Buschmann, MD*‖

Freiburg and Berlin, Germany; and Utrecht and Amsterdam, the Netherlands

OBJECTIVES The objective of this study was to test the potential of aspirin and clopidogrel to influence collateral artery growth (arteriogenesis).

BACKGROUND Aspirin and clopidogrel are antiplatelet agents commonly used in the treatment of ischemic cardiovascular disease. Both inhibit platelet aggregation; however, they differ mechanistically because aspirin acts via cyclooxygenase (COX) inhibition, while clopidogrel noncompetitively antagonizes the $P2Y_{12}$ adenosine diphosphate receptor. We hypothesized that aspirin, due to its anti-inflammatory effects through inhibition of COX activity could inhibit arteriogenesis. Given that clopidogrel does not affect COX activity, it would be less likely to interfere with collateral artery growth.

METHODS Fifty-four New Zealand White rabbits received either saline, aspirin (10 mg/kg), or clopidogrel (10 mg/kg) for seven days after femoral artery ligation. Maximal collateral conductance was assessed with fluorescent microspheres under maximal vasodilation; cellular migration and proliferation (Ki-67) was evaluated by quantitative immunohistology.

RESULTS Collateral conductance was significantly reduced by aspirin treatment, whereas clopidogrel had a neutral effect (saline: 0.94 ± 0.04; clopidogrel: 0.94 ± 0.05; aspirin: 0.64 ± 0.03 $ml \cdot min^{-1} \cdot 100\ mm\ Hg^{-1} \cdot g^{-1}$; $p < 0.001$). Ki-67 proliferation indexes were consistent with these results (saline: 23.1 ± 2.9%; clopidogrel: 23.5 ± 1.1%; aspirin: 19.2 ± 1.1% Ki-67–positive cells). Immunohistochemistry showed COX expression in collateral arteries and a significantly decreased monocyte/macrophage accumulation in the perivascular tissue after aspirin treatment. Cell adhesion molecule expression on monocytes after activation was significantly reduced by aspirin, which might explain the reduced migratory ability.

CONCLUSIONS In summary, clopidogrel had a neutral effect on natural arteriogenesis. Aspirin significantly inhibited collateral artery growth, probably due to its anti-inflammatory effect. Additional studies are needed to substantiate these results before translation into clinical practice. (J Am Coll Cardiol 2005;46:994–1001) © 2005 by the American College of Cardiology Foundation

As a natural compensation mechanism upon arterial occlusion, a preexisting network of collateral anastomoses is recruited and develops into functional collateral arteries. This process is referred to as arteriogenesis and provides the ischemic territory with nutrient blood flow (1). A decisive step in arteriogenesis is the increase in the pressure gradient along the arteriolar anastomoses (prestenotic minus poststenotic pressure), once a stenosis in the main feeding artery becomes hemodynamically relevant. The increase in net forward blood flow leads to increased shear forces acting on the endothelium of collateral arteries. This sustained increase in shear stress within newly recruited collateral arteries leads to expression and presentation of cell adhesion molecules (e.g., intracellular adhesion molecule-1, vascular cell adhesion molecule-1) (2) and up-regulation of chemokines and colony stimulating factors by the endothelium. The secreted cytokines attract monocytes, which then adhere to endothelial cell adhesion molecules, transmigrate, and accumulate in the perivascular tissue of proliferating collateral arteries (3,4). After infiltration, the monocytes mature to macrophages, producing numerous cytokines and degrading enzymes, thereby creating the controlled inflammation necessary to remodel an arteriole into an artery (3,5). This scenario is in large part similar to the processes occurring during inflammation, and the factors involved are often identical (6,7).

Compounds inhibiting platelet aggregation (e.g., aspirin, clopidogrel) have been shown to successfully reduce cardiovascular mortality in patients suffering from coronary heart disease (8). In particular, aspirin has been shown to improve clinical outcome in numerous studies. Aspirin belongs to the

From the *Research Group for Arteriogenesis, Department of Cardiology, University of Freiburg, Freiburg, Germany; †Perfusion Technologies GmbH, Freiburg, Germany; ‡Department of Experimental Cardiology, UMC, University of Utrecht, Utrecht, the Netherlands; §Department of Cardiology, AMC, University of Amsterdam, Amsterdam, the Netherlands; and ‖Humboldt University, Center for Cardiovascular Research, Charité, Berlin, Germany. This work was supported by Bristol-Myers-Squibb Germany.

Manuscript received August 4, 2004; revised manuscript received January 20, 2005, accepted February 14, 2005.

Abbreviations and Acronyms

ADP = adenosine diphosphate
COX = cyclooxygenase
Mac-1 = membrane attack complex-1
LPS = lipopolysaccharide
LFA-1 = leukocyte functional antigen-1
MCP = monocyte chemoattractant protein
NSAID = non-steroidal anti-inflammatory drug

group of non-steroidal anti-inflammatory drugs (NSAIDs) that have been shown to inhibit leukocyte accumulation during inflammatory diseases (9–11). Although the mechanism of this anti-inflammatory action is not completely understood, inhibition of the cyclooxygenases (COX) has been proven to be crucial. Aspirin is a potent irreversible inhibitor of both COX-1 and COX-2. In addition to its inhibitory effect on leukocyte migration (12–14), it further inhibits subsequent leukocyte activation (15). In contrast, clopidogrel acts as a noncompetitive antagonist of the $P2Y_{12}$ adenosine diphosphate (ADP) receptor on platelets and does not display comparable anti-inflammatory properties.

Because arteriogenesis depends on monocyte migration and activation, we hypothesized that treatment with aspirin potentially attenuates arteriogenesis after arterial occlusion, whereas clopidogrel might not.

METHODS

This study was performed after securing appropriate institutional approvals. It conforms to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996). After ligation of the right femoral artery as previously described (16), 54 New Zealand White rabbits of comparable weight (3.0 ± 0.2 kg) and age were randomly assigned to one of three groups (n = 18 each). Assignment to the groups was done by an observer blinded to the purpose and the treatment options of the study. Group 1 consisted of animals receiving normal saline orally, whereas animals of group 2 received clopidogrel (obtained from Bristol-Myers-Squibb, Munich, Germany) per os once daily in a dose of 10 mg/kg body weight via gavage). Group 3 received aspirin (acetylic salicylic acid) 10 mg/kg body weight/day analogous to group 2. These doses have been shown to effectively inhibit platelet aggregation to a comparable degree in the rabbit in previous studies (17–19). No animal suffered gangrene, necrosis, or gross impairment of hind limb function after the initial operation. There was no observable difference in postoperative behavior or body weight between any groups at any time point.

During the observation period, blood samples were withdrawn from six animals per group for assessment of leukocyte activation. Seven days after femoral artery ligation, the animals were again anesthetized for either postmortem angiography (n = 6 each), hemodynamic measurements (n = 6 each), or preparation of histological samples (n = 6 each). Data collection and analysis was performed by an observer blinded to the group assignment until completion of the study.

Postmortem angiograms. After maximal vasodilatation with adenosine, legs were perfused with PBS in a warmed waterbath (37°C) for 1 min at 80 mm Hg followed by perfusion for 8 min at 80 mm Hg with contrast medium based on bismuth and gelatin (20). Subsequently, the contrast medium was allowed to gel by placing the limbs on crushed ice for 45 min.

Collateral arteries were classified according to Longland (21) into stem, midzone, and reentry. Collateral vessels were marked after counting to be certain that no vessel was counted twice.

Hemodynamic measurements. Collateral conductance measurements were performed as previously described (16). A pump-driven shunt between the left carotid artery and abdominal aorta was installed by cannulating both vessels. To ensure proper mixing of the microspheres with the perfusate, a mixing chamber was serially installed into the shunt system. Prestenotic blood pressure was measured at the place of the aortic cannula insertion. Poststenotic blood pressure was measured by cannulating the saphenous artery just above the ankle. The left femoral artery was cannulated for withdrawal of blood flow reference samples for subsequent calculation of the specific blood flows to each tissue sample. This resulted in an acute occlusion of the left femoral artery, and perfusion data of the left hind limb was therefore used as an internal control, ensuring comparability of the obtained data. Using a roller-pump, six different pressure levels were maintained under continuous flow and pressure monitoring. Maximal vasodilation was achieved by continuous adenosine infusion into the shunt system (1 mg/kg/min). At each pressure level, fluorescent microspheres (Molecular Probes, Leiden, the Netherlands) with a different label (in order of injection: scarlet, crimson, red, blue-green, orange, and yellow-green, $2 \cdot 10^6$ each) were injected.

Tissue samples for the evaluation of collateral-dependent flow were harvested after weighing the muscles they were derived from and then weighed and further processed for flow cytometric quantification of microspheres per sample. Flow to each tissue sample was then calculated from the number of microspheres in the samples and the respective amount of microspheres in the reference sample. Based on the weight of the tissue samples, perfusion was expressed as ml/min/g tissue. For a better comparison with other studies, the total tissue mass depending on collateral perfusion was weighed and multiplied with the perfusion values per gram, which thus indicates the total conductance of the collateral network. The resulting collateral blood flows were plotted against their respective pressure levels (pressure gradient of prestenotic minus poststenotic pressure). Collateral conductance equals the slope of the resulting curve of the six different pressure-flow relations and reflects the change in collateral flow per increase of the pressure gradient. Slope

was calculated by least squares with R^2 values of 0.89 ± 0.01.

Leukocyte activation testing. At day 1, 3, and 5 after the initial operation, blood was withdrawn from animals of each group in heparin-coated tubes to analyze leukocyte activation. Directly after the withdrawal, whole blood was stained for CD11a, CD11b, and CD18 (all Serotec, Oxford, United Kingdom) using specific mouse anti-rabbit antibodies. For identification of monocytes, blood samples were furthermore stained with a phycoerythrin-conjugated anti-CD14 antibody (DAKO, Glastrup, Denmark). The blood samples were then divided into three smaller samples, from which the first one was left untreated and served as the control. Samples two and three were stimulated with lipopolysaccharide (LPS) (200 ng/ml) and monocyte chemoattractant protein (MCP)-1 (200 ng/ml), respectively. After addition of the factors, samples were incubated at 37°C in a 5% CO_2 atmosphere for 2 h. Subsequent to the incubation period, samples were stained as described above for examination of surface molecule expression. The influence of the previous treatment on the activation potency of leukocytes was expressed as the ratio of stimulated to nonstimulated blood.

Histological examinations of in vivo cell migration. Frozen tissue sections (5 μm) were stained for Ki-67 (mouse anti-rat Ki-67, Clone MIB-5, DAKO) to quantify the proliferative index of vascular smooth muscle cells (Sigma Chemical Company, St. Louis, Missouri) and the endothelium. Thirty sections in 400-μm intervals from the vastus intermedius quadriceps per animal were analyzed by a blinded observer under 400-fold magnification. The proliferative index of each vessel was calculated as the percentage of Ki-67–positive cells within the growing collateral arteries. Only growing collateral arteries are positively marked for the proliferation marker Ki-67, whereas vascular smooth muscle cells from quiescent "normal" arteries remain negative.

To examine and quantify the number of transmigrated leukocytes, tissue sections were performed as above and stained with a mouse anti-rabbit CD11b (Mouse anti-Rabbit CD11b, Clone 198, Serotec) antibody, staining granulocytes and monocytes/macrophages. Only cells definitely showing a positive stain and an identifiable nucleus were counted in one microscopic field around the collateral artery at 200-fold magnification by a blinded observer. Additionally, monocytes/macrophages were stained with a mouse anti-human CD68 antibody (Monoclonal Mouse anti-Human CD68, Macrophage, Clone EBM11, DAKO), which has been shown to cross-react with rabbit monocytes/macrophages previously. Again, only cells with positive staining around a nucleus were identified and counted as macrophages. Numbers were expressed per mm^2, calculated from the area of the microscopic field.

Expression of COX-1 was assessed by staining with a goat anti-human COX-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California) recognizing the carboxy terminus of COX-1. Double staining for CD11b as mentioned above was performed to identify the identity of perivascular COX-1–positive cells.

Statistical analysis. Results are expressed as mean ± SEM. All statistical analyses between treatment groups and control group were performed with SigmaStat software (SPSS Inc., Chicago, Illinois) using one-way analysis of variance and post-hoc Bonferroni correction (n = 3 comparisons for each analysis).

RESULTS

Postmortem angiograms. Postmortem angiograms performed after seven days showed numerous collateral arteries running from the arteria circumflexa femoris and the arteria profunda femoris, thereby bypassing the stenosis caused by the ligation of the femoral artery. There was no significant difference between any of the groups, as the number of angiographically detectable collateral arteries was not measurably altered by any treatment regimen (saline: 16.7 ± 1.2; clopidogrel: 17.3 ± 1.2; aspirin: 16.2 ± 2.0) (Fig. 1).

Hemodynamic measurements. Collateral conductance seven days after ligation of the right femoral artery showed a 5-fold increase as compared to acute occlusion of the left femoral artery. Daily oral administration of clopidogrel at 10 mg/kg did not affect collateral conductance (saline: 0.94 ± 0.04 $ml \cdot min^{-1} \cdot 100\ mm\ Hg^{-1} \cdot g^{-1}$, clopidogrel: 0.94 ± 0.05 $ml \cdot min^{-1} \cdot 100\ mm\ Hg^{-1} \cdot g^{-1}$; p = NS). In contrast, oral treatment with aspirin using the same dosage led to a significant reduction in collateral conductance compared to untreated as well as to clopidogrel-treated animals (aspirin: 0.64 ± 0.03 $ml \cdot min^{-1} \cdot 100\ mm\ Hg^{-1} \cdot g^{-1}$; saline vs. aspirin $p < 0.001$; clopidogrel vs. aspirin $p < 0.001$). Furthermore, to allow better comparisons with other studies, collateral conductance for the total tissue mass depending on collateralization was calculated (Fig. 2).

Leukocyte activation. Immediately after withdrawal of the blood samples, no significant difference in membrane attack complex-1 (Mac-1) and leukocyte functional antigen-1 (LFA-1) expression on monocytes and granulocytes was detected between untreated, aspirin-treated, and clopidogrel-treated animals. However, the relative increase of Mac-1 (CD11b) and LFA-1 (CD11a) expression as markers of cellular activation was significantly reduced by prior aspirin treatment as compared to leukocytes from untreated animals. This observation remained constant during the treatment period. In contrast to the findings in aspirin-treated animals, clopidogrel treatment did not affect the efficacy of monocyte activation by MCP-1 (CD11a expression: saline: 1.27 ± 0.02; clopidogrel: 1.31 ± 0.04; aspirin: 1.18 ± 0.03; $p < 0.05$) (CD11b expression: saline: 1.26 ± 0.04; clopidogrel: 1.29 ± 0.03; aspirin: 1.22 ± 0.03, p = NS) and LPS (CD11a expression: saline: 1.38 ± 0.04; clopidogrel: 1.39 ± 0.03; aspirin: 1.15 ± 0.03; $p < 0.05$) (CD11b expression: saline: 1.50 ± 0.03; clopidogrel: 1.42 ± 0.03; aspirin: 1.36 ± 0.04; $p < 0.05$ for aspirin vs. saline; p = NS for clopidogrel vs. aspirin).

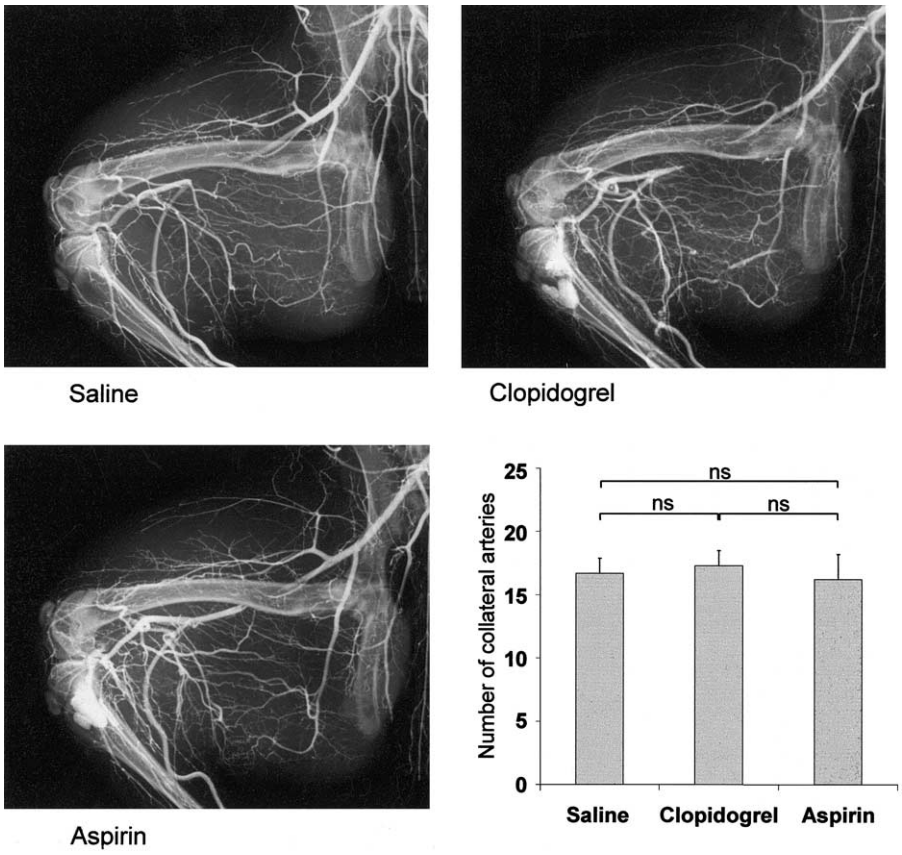

Figure 1. Postmortem angiograms one week after femoral artery ligation. Neither aspirin nor clopidogrel treatment affected angiographic appearance and number of collateral arteries.

Histological examination of in vivo cell migration. Proliferating collateral arteries showed positive staining for COX-1 in the endothelium and a higher expression in migrated cells. Double staining for CD11b identified transmigrated leukocytes as the source of the perivascular COX-1 expression (Fig. 3A). In normal arterial vessels not undergoing proliferation in the control hind limb, COX-1 could only be found in endothelial cells.

Quantitative assessment of leukocyte migration into the perivascular space of the growing collateral arteries showed comparable numbers of CD68-positive monocytes/macrophages in untreated and clopidogrel-treated animals.

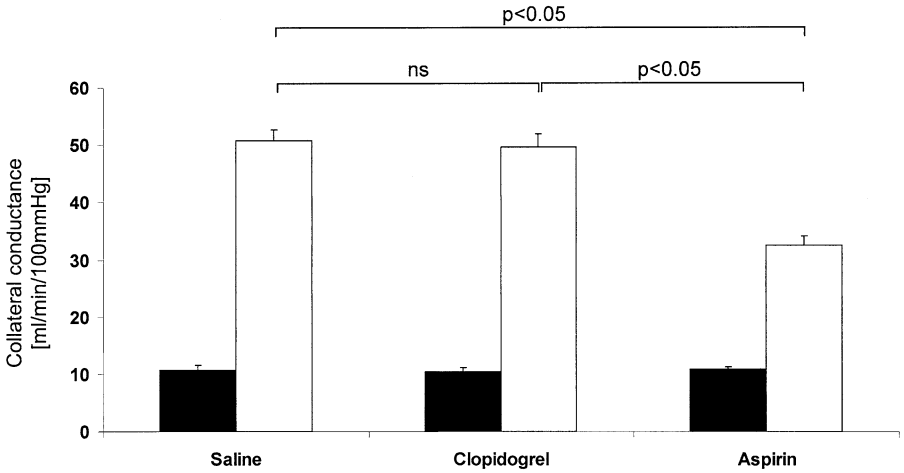

Figure 2. Collateral conductance measurements for the total tissue mass that depends on the collateral network seven days after femoral artery ligation **(open bars)**. Aspirin treatment significantly reduced collateral conductance, whereas clopidogrel treatment did not functionally affect arteriogenesis (saline: 50.7 ± 1.9 ml/min/100 mm Hg; clopidogrel: 49.5 ± 2.4 ml/min/100 mm Hg; aspirin: 32.6 ± 1.6 ml/min/100 mm Hg). The contralateral hind limb that was subjected to acute occlusion of the femoral artery served as an internal control **(solid bars)**.

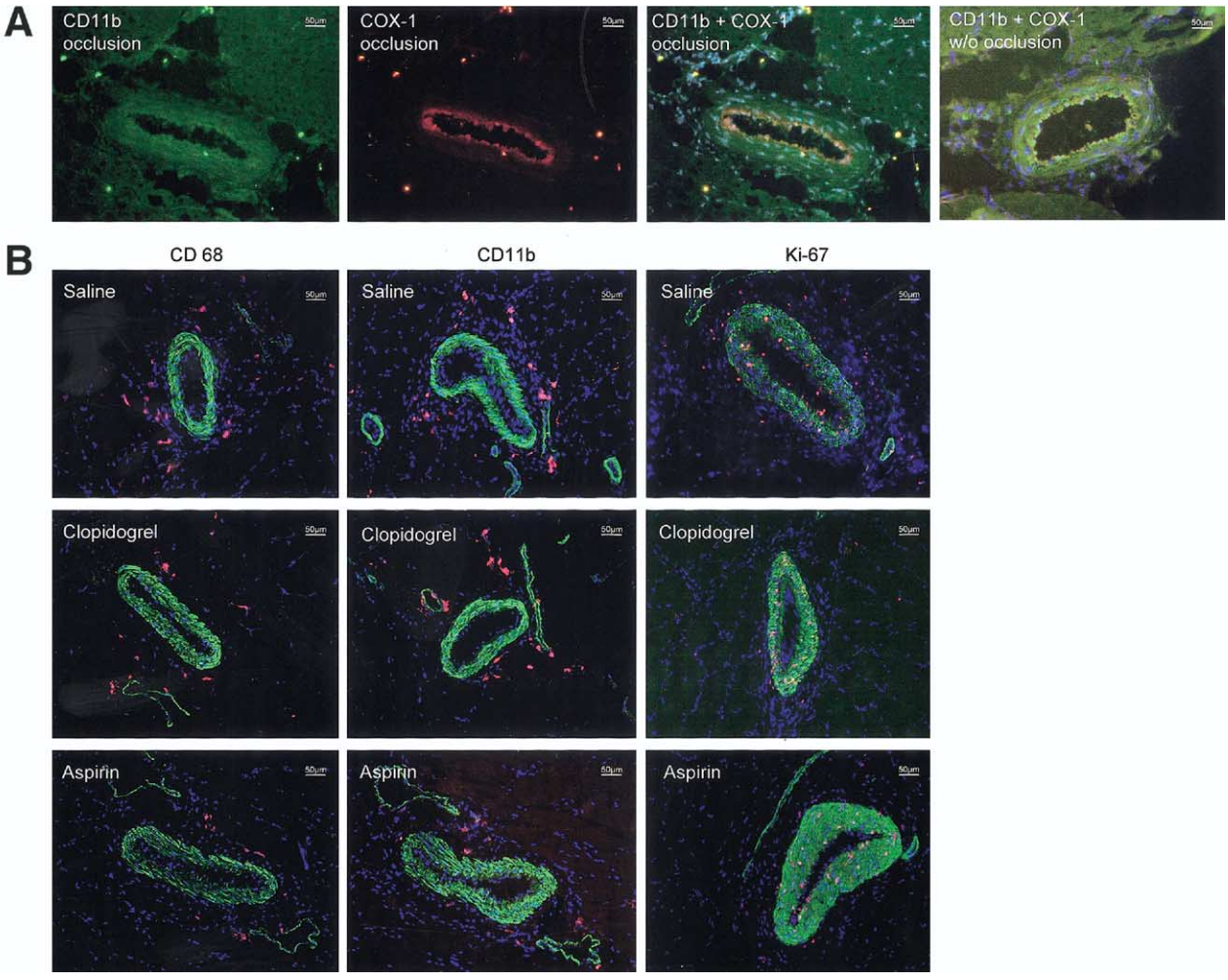

Figure 3. (A) Staining for cyclooxygenase (COX)-1 **(red)** indicated expression in the endothelium of quiescent arteries in the control hind limb. In proliferating collateral arteries, transmigrated CD11b-positive leukocytes **(green)** reveal a strong positive stain and serve as additional sources of COX-1. **(B)** Immunohistological staining for vascular smooth muscle cells **(green)** and accumulating monocytes/macrophages **(red)** in the surrounding tissue of the proliferating collateral arteries **(first two columns)**. Proliferating cells **(third column)** stained for the Ki-67 antigen **(red)** are mainly localized in the media of the growing vessels. Vascular smooth muscle cells are stained **green** and nuclei appear **blue**. Aspirin treatment significantly reduced leukocyte migration and proliferation; clopidogrel-treated animals showed comparable results as saline-treated animals.

The number of transmigrated monocytes/macrophages in aspirin-treated animals was significantly reduced compared to the other groups (saline: 27.0 ± 3.3; clopidogrel: 22.9 ± 1.3; aspirin: 17.5 ± 0.4 CD68-positive cells mm^2) (Fig. 3B, left column). Comparable results were obtained for the quantity of CD11b-positive leukocytes (monocytes/macrophages and granulocytes). While clopidogrel treatment did not affect accumulation of CD11b-positive cells, treatment with aspirin significantly reduced leukocyte migration (saline: 33.1 ± 2.3; clopidogrel: 29.6 ± 1.9; aspirin: 18.5 ± 1.3 CD11b-positive cells/mm^2) (Fig. 3B, middle column).

Proliferation indexes. Ki-67 staining showed growth and proliferation of arteriolar anastomoses to functional collateral arteries, whereas "normal" muscle feeding vessels remained negative. Aspirin treatment significantly reduced the proliferation index (Ki-67–positive cells vs. total number of cells) in the media of collateral vessels as compared to the control and the clopidogrel group (saline: 23.1 ± 1.2%; clopidogrel: 23.5 ± 0.4%; aspirin: 19.2 ± 0.5% Ki-67–positive vascular smooth muscle cells) (Fig. 3B). In concordance with previous reports (22), this correlated well with proliferation of the endothelium in these vessels (saline: 19.4 ± 0.4%; clopidogrel: 19.6 ± 0.4%; aspirin: 17.6 ± 0.3% Ki-67–positive endothelial cells).

DISCUSSION

Ischemic events affecting the cerebral, coronary, and peripheral arteries are different manifestations of a common pathophysiological process, namely atherothrombosis, or thrombus formation superimposed on preexisting atherosclerotic plaque (23). Aspirin is thought to prevent thrombotic vascular occlusion by inhibiting platelet aggregation, and is widely prescribed for this purpose. More recently, novel compounds (e.g., clopidogrel) have been developed to inhibit platelet aggregation, although by different pharmacodynamic mechanisms. Aspirin irreversibly and unselec-

tively blocks COX-1 and -2, whereas clopidogrel (i.e., its active metabolite) acts as a noncompetitive antagonist of the $P2Y_{12}$ ADP receptor on platelets. Because platelet inhibitors have become the standard medications for the treatment of patients with cardiovascular diseases, this study focused on the role of aspirin and clopidogrel on collateral artery growth (arteriogenesis). Our hypothesis was further supported by the observation of the Clopidogrel Versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) investigators, who described a relative risk reduction for stroke, myocardial infarction, and death due to vascular causes of 8.7% with clopidogrel compared to aspirin in more than 19,000 patients with recent symptomatic atherosclerotic disease (24,25).

Nonsteroidal anti-inflammatory drugs and their effect on vessel growth, in particular their influence on angiogenesis (i.e., formation of new capillary networks) have been the subject of numerous studies. Although recent studies showed comparable antiangiogenic properties of aspirin and other NSAIDs in different models, the underlying mechanisms have not yet been clarified (26–29). However, arteriogenesis differs from angiogenesis: collateral vessels are preexisting rather than newly built vessels (30), and their growth is not dependent on ischemia (4,31), but is mainly induced by increased hemodynamic shear forces.

To the best of our knowledge, this is the first study to report an inhibitory effect of aspirin on arteriogenesis. The rabbit hind limb model is well-established and widely used to examine collateral artery growth in the peripheral circulation and offers the possibility of hemodynamic measurements with fluorescent microspheres. Moreover, the latter constitutes the "gold standard" in measuring tissue perfusion (32). Compared to other means of assessing vascular growth processes (e.g., blood flow at rest) this method has been shown to be more accurate in measuring collateral artery growth recently (22).

In our study collateral conductance (as the functional parameter of collateral artery growth) was significantly decreased by daily oral administration of aspirin. Compared to placebo-treated control animals, conductance was reduced by ~40%. Quantitative immunohistochemistry for vascular proliferation (Ki-67) provided additional evidence, because aspirin-treated animals showed significantly lower numbers of Ki-67–positive cells in the growing collateral vessels. The significant reduction in perfusion could not be deducted from postmortem angiograms. Due to the law of Hagen-Poiseuille, very small changes in vessel diameter result in high changes in blood flow; therefore collateral vessel density in angiograms does not necessarily correlate with collateral flow. Furthermore, previous studies have shown that angiographic scoring does not reflect tissue perfusion as assessed by microsphere techniques (33). The observed differences in conductance (~40%) reflect diameter changes of <10%, which are impossible to detect using the angiographic techniques employed in this study. Therefore, unlike previous studies with proarteriogenic compounds, no differences between treatment groups could be detected by quantification of postmortem angiographies. To detect these minor changes in diameter, more advanced but limited available imaging methods are needed. Another explanation for the observed change in collateral flow might be that the vasodilatory response is affected by the treatment. This was ruled out by simultaneously measuring collateral conductance of the contralateral hind limbs that underwent an acute occlusion at the time of perfusion measurement, which showed no significant differences between the groups (Fig. 2). The dose of adenosine has been shown to ensure complete vasodilation in the rabbit hind limb in different studies, abolishing any further vasodilatory response (e.g., upon reactive hyperemia). Furthermore, adenosine was evaluated versus other vasodilatory compounds (e.g., papaverine) (data not shown).

Staining for COX-1 in proliferating collateral arteries identified transmigrated, CD11b-positive leukocytes as the major source of COX-1 apart from the endothelium, which also exhibited low COX-1 expression in normal arteries not undergoing proliferation. This is consistent with data from atherosclerotic patients colocalizing COX expression with macrophages (34).

As previously described, monocytes/macrophages are the key mediators of arteriogenesis, in particular during early stages (2). In aspirin-treated rabbits, the number of CD68-positive macrophages in the adventitial tissue was significantly reduced. As described above, the mechanisms underlying arteriogenesis are to a large extent similar to mechanisms responsible for inflammatory processes. Previous studies have shown that aspirin significantly inhibits leukocyte/monocyte adhesion to endothelium (35–37) and transendothelial migration (15,38), the first mandatory steps of the arteriogenic response in early phases.

Monocyte transmigration is mediated by different cell adhesion molecules. Leukocytes (e.g., monocytes) attach via interaction of the heterodimers CD18/CD11b (Mac-1) and CD18/CD11a (LFA-1) with intercellular adhesion molecule-1 on the endothelium (39). Mac-1 is recognized as an early marker of monocyte activation and is up-regulated by different activating factors (e.g., LPS, MCP-1). We therefore tested whether monocytes from aspirin-treated animals show an activation response to LPS and MCP-1 comparable to monocytes from untreated or clopidogrel-treated animals. The increase in CD11b expression after in vitro stimulation of aspirin-treated monocytes was significantly lower than the activation potential of untreated monocytes. This might explain the decreased numbers of migrated monocytes/macrophages after aspirin treatment. As a potential consequence, the deficit in growth factors released by macrophages leads to reduced proliferation and a reduced functionality of the collateral network (i.e., a significant inhibition and deceleration of arteriogenesis).

From the clinical point of view, it is reasonable to speculate that a potential reduction of adaptive arteriogenesis with aspirin treatment might have a deleterious effect in

the setting of ischemic cardiovascular disease, compared to other compounds with equal antithrombotic activity, but without this unwanted side effect.

Study limitations. The results of this study are based on doses of aspirin and clopidogrel higher than those given in clinical practice. This might partially explain the prominent effect of aspirin treatment. Furthermore, we have not tested the antiplatelet properties of the substances during our experiments. Due to the different mechanisms of action, a direct comparison of the antiplatelet effect of clopidogrel and aspirin in the rabbit is not possible and requires different inducers (e.g., ADP, arachidonic acid) of platelet aggregation. However, the used doses have been proven previously to be of comparable efficacy regarding inhibition of platelet aggregation in rabbits (19).

Conclusions. In summary, this is the first study demonstrating a direct inhibitory effect of aspirin on collateral artery growth. Clopidogrel, acting via mechanisms different than COX inhibition, did not affect the natural arteriogenic response. The possible clinical relevance of this observation will be tested prospectively in the ongoing ARTeriogenesis NETwork (ART.NET)-1 trial. In this multicenter study, the effects of physical training, aspirin, or clopidogrel on the walking capacity of patients with stage II peripheral arterial disease will be evaluated (n = 125 patients/group).

Reprint requests and correspondence: Dr. Imo E. Hoefer, UMC Utrecht, G02.523, Heidelberglaan 100, NL-3584 CX Utrecht, the Netherlands. E-mail: i.hoefer@azu.nl.

REFERENCES

1. Buschmann I, Schaper W. Arteriogenesis versus angiogenesis: two mechanisms of vessel growth. News Physiol Sci 1999;14:121–5.
2. Scholz D, Ito W, Fleming I, et al. Ultrastructure and molecular histology of rabbit hindlimb collateral artery growth (arteriogenesis). Virchows Archiv 2000;436:257–70.
3. Schaper J, Koenig R, Franz D, Schaper W. The endothelial surface of growing coronary collateral arteries. Intimal margination and diapedesis of monocytes. A combined SEM and TEM study. Virchows Arch A (Pathol Anat) 1976;370:193–205.
4. Deindl E, Buschmann I, Hoefer I, et al. Role of ischemia and of hypoxia-inducible genes in arteriogenesis after femoral artery occlusion in the rabbit. Circ Res 2001;89:779–86.
5. Schaper W, Jageneau A, Xhonneux R. The development of collateral circulation in the pig and dog heart. Cardiology 1967;51:321–35.
6. Joussen AM, Poulaki V, Mitsiades N, et al. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. FASEB J 2002;16:438–40.
7. Hoefer IE, van Royen N, Rectenwald JE, et al. Direct evidence for tumor necrosis factor-alpha signaling in arteriogenesis. Circulation 2002;105:1639–41.
8. Merz CN, Rozanski A, Forrester JS. The secondary prevention of coronary artery disease. Am J Med 1997;102:572–81.
9. Zak-Nejmark T, Jankowska R, Malolepszy J, Kraus-Filarska M, Nadobna G, Nowak IA. Modulation of adhesion and chemotaxis of human neutrophils by cortisol, transforming growth factor-beta and antiinflammatory drugs. J Investig Allergol Clin Immunol 1998;8:346–52.
10. Pillinger MH, Capodici C, Rosenthal P, et al. Modes of action of aspirin-like drugs: salicylates inhibit erk activation and integrin-dependent neutrophil adhesion. Proc Natl Acad Sci U S A 1998;95:14540–5.
11. Diaz-Gonzalez F, Gonzalez-Alvaro I, Campanero MR, et al. Prevention of in vitro neutrophil-endothelial attachment through shedding of L-selectin by nonsteroidal antiinflammatory drugs. J Clin Invest 1995;95:1756–65.
12. Spisani S, Vanzini G, Traniello S. Inhibition of human leucocytes locomotion by anti-inflammatory drugs. Experientia 1979;35:803–4.
13. Borel JF. Effect of some drugs on the chemotaxis of rabbit neutrophils in vitro. Experientia 1973;29:676–8.
14. Borel JF, Feurer C. Chemotaxis of rabbit macrophages in vitro: inhibition by drugs. Experientia 1975;31:1437–9.
15. Gerli R, Paolucci C, Gresele P, et al. Salicylates inhibit adhesion and transmigration of T lymphocytes by preventing integrin activation induced by contact with endothelial cells. Blood 1998;92:2389–98.
16. Hoefer I, van Royen N, Buschmann I, Piek J, Schaper W. Time course of arteriogenesis following femoral artery occlusion in the rabbit. Cardiovasc Res 2001;49:609–17.
17. Golino P, Ambrosio G, Pascucci I, Ragni M, Russolillo E, Chiariello M. Experimental carotid stenosis and endothelial injury in the rabbit: an in vivo model to study intravascular platelet aggregation. Thromb Haemost 1992;67:302–5.
18. Kawano KI, Fujishima K, Ikeda Y, Kondo K, Umemura K. ME3277, a GPIIb/IIIa antagonist reduces cerebral infarction without enhancing intracranial hemorrhage in photothrombotic occlusion of rabbit middle cerebral artery. J Cereb Blood Flow Metab 2000;20:988–97.
19. Herbert JM, Dol F, Bernat A, Falotico R, Lale A, Savi P. The antiaggregating and antithrombotic activity of clopidogrel is potentiated by aspirin in several experimental models in the rabbit. Thromb Haemost 1998;80:512–8.
20. Fulton WFM. The Coronary Arteries. Springfield, IL: Charles C. Thomas, 1965.
21. Longland CJ. The collateral circulation of the limb. Ann R Coll Surg Engl 1953;13:161–4.
22. Schirmer SH, Buschmann IR, Jost MM, et al. Differential effects of MCP-1 and leptin on collateral flow and arteriogenesis. Cardiovasc Res 2004;64:356–64.
23. Ringleb PA, Bhatt DL, Hirsch AT, Topol EJ, Hacke W. Benefit of clopidogrel over aspirin is amplified in patients with a history of ischemic events. Stroke 2004;35:528–32.
24. CAPRIE Steering Committee. A randomised, blinded, trial of Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE). Lancet 1996;348:1329–39.
25. Harker LA, Boissel JP, Pilgrim AJ, Gent M, CAPRIE Steering Committee and Investigators. Comparative safety and tolerability of clopidogrel and aspirin: results from CAPRIE. Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events. Drug Saf 1999;21:325–35.
26. Sharma S, Ghoddoussi M, Gao P, Kelloff GJ, Steele VE, Kopelovich L. A quantitative angiogenesis model for efficacy testing of chemopreventive agents. Anticancer Res 2001;21:3829–37.
27. Szabo IL, Pai R, Soreghan B, et al. NSAIDs inhibit the activation of egr-1 gene in microvascular endothelial cells. A key to inhibition of angiogenesis? J Physiol Paris 2001;95:379–83.
28. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998;93:705–16.
29. Jones MK, Wang H, Peskar BM, et al. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med 1999;5:1418–23.
30. Herzog S, Sager H, Khmelevski E, Deylig A, Ito WD. Collateral arteries grow from preexisting anastomoses in the rat hindlimb. Am J Physiol Heart Circ Physiol 2002;283:H2012–20.
31. Ito WD, Arras M, Winkler B, Scholz D, Htun P, Schaper W. Angiogenesis but not collateral growth is associated with ischemia after femoral artery occlusion. Am J Physiol 1997;273:H1255–65.
32. Unger E. Experimental evaluation of coronary collateral development. Cardiovasc Res 2001;49:497–506.
33. Fuchs S, Shou M, Baffour R, Epstein SE, Kornowski R. Lack of correlation between angiographic grading of collateral and myocardial perfusion and function: implications for the assessment of angiogenic response. Coron Artery Dis 2001;12:173–8.

34. Schonbeck U, Sukhova GK, Graber P, Coulter S, Libby P. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am J Pathol 1999;155:1281–91.
35. Krumholz W, Szalay G, Ogal H, Menges T. Effect of migraine medications on monocyte chemotaxis (in German). Anaesthesiol Reanim 2000;25:102–4.
36. Fricchione GL, Bilfinger TV, Stefano GB. Aspirin inhibits granulocyte and monocyte adherence to saphenous vein endothelia in a process not mediated by nitric oxide. Int J Cardiol 1998;64: S29–33.
37. Weber C, Erl W, Pietsch A, Weber PC. Aspirin inhibits nuclear factor-kappa B mobilization and monocyte adhesion in stimulated human endothelial cells. Circulation 1995;91:1914–7.
38. Pierce JW, Read MA, Ding H, Luscinskas FW, Collins T. Salicylates inhibit I kappa B-alpha phosphorylation, endothelial-leukocyte adhesion molecule expression, and neutrophil transmigration. J Immunol 1996;156:3961–9.
39. Hoefer IE, van Royen N, Rectenwald JE, et al. Arteriogenesis proceeds via ICAM-1/Mac-1-mediated mechanisms. Circ Res 2004; 94:1179–85.