Mechanisms of arteriogenesis: from cellular adhesion to therapeutic stimulation
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DIVERGENT EFFECTS OF ANTI-PLATELET DRUGS ON ARTERIOGENESIS AFTER FEMORAL ARTERY OCCLUSION IN THE RABBIT HINDLIMB

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Abstract

The progressive stenosis of a main feeding artery leads to a significant increase in flow and shear forces in pre-existent arteriolar anastomoses bypassing the site of arterial obstruction. Hence the endothelium of collateral arterial pathways is activated and expresses cell adhesion molecules and cytokines to attract circulating leukocyte. Monocytes attach to the vascular wall, transmigrate and produce a variety of growth factors and enzymes necessary for the rapid proliferation and remodeling of small resistance anastomoses into large conduction arteries. Thus this process, arteriogenesis, is characterized by an increase numbers in inflammatory cells (e.g. monocytes), cytokines (e.g. TNF-alpha), and matrix degrading enzymes (e.g. MMPs).

The aim of the current study therefore was to investigate the influence of platelet inhibitors on this important rescue mechanism for cardiovascular disease. Fifty-four New Zealand White rabbits received either solvent, ASA (10 mg/kg) or clopidogrel (10 mg/kg) for a period of seven days after ligation of the right femoral artery. Collateral conductance measurements under maximal vasodilation using fluorescent microspheres showed a significant inhibition of arteriogenesis by ASA treatment, whereas clopidogrel acted neutral (solvent: 50.70 ± 11.52; clopidogrel: 49.53 ± 14.22; ASA: 32.55 ± 9.54 ml/min/100mmHg; p<0.05). Ki-67 proliferation indices confirmed these results (solvent 23.10 ± 2.94; clopidogrel 23.53 ± 1.08; ASA 19.15 ± 1.12 % Ki-67 positive cells). Quantitative immunohistochemistry showed a significantly lower number of monocytes/macrophages in the surrounding tissue of collateral vessels from ASA treated animals. FACS-analysis indicated a reduction of cell adhesion molecule expression on blood monocytes after activation under ASA treatment, which might explain the reduced migratory ability. In summary, clopidogrel acts neutral on natural arteriogenesis. ASA significantly inhibited collateral artery growth, probably due to its anti-inflammatory effect. This insight might be of importance for the choice of the appropriate platelet inhibitor for patients with occlusive arterial disease.
Introduction

Cardiovascular disease is the commonest cause of death worldwide. Cardiovascular diseases, together with cerebrovascular disease claim more lives each year than the next 7 leading causes of death combined. In most cases, cardiovascular disorders are caused by the development of an atherosclerotic lesion and subsequent stenosis of the artery. Moreover, in the vast majority of patients atherosclerotic plaques may rupture and the following adhesion of platelets leads to thrombus formation superimposed on preexisting atherosclerosis (atherothrombosis).

Compounds inhibiting platelet aggregation have been shown to successfully reduce the cardiovascular mortality in patients suffering from coronary heart disease. One of the first drugs showing a potent platelet inhibition, Acetylsalicylic Acid (ASA), has been proven to improve the outcome in numerous clinical studies. In particular, following invasive procedures for the restoration of blood flow in occluded arteries (PTA, PTCA, CABG), an efficient platelet inhibition with a combination of different anti-platelet drugs (e.g. ASA + clopidogrel) is commonly used to decrease the rate of re-stenosis.

In most patients also a natural compensation mechanism for arterial obstruction can be observed. Upon arterial occlusion, a pre-existing network of collateral anastomoses is recruited and develops into functional collateral conduit arteries. This process is referred to as arteriogenesis and provides the ischemic territory with nutrient blood flow.

A decisive step in arteriogenesis is the increase in the pressure gradient along the arteriolar anastomoses (pre-stenotic minus post-stenotic 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 endothelial layers of collateral arteries.

Previous studies have shown that a sustained increase in shear stress within newly recruited collateral arteries leads to the expression and presentation of cells adhesion molecules (e.g. ICAM-1, VCAM-1) and upregulation of chemokines as well as colony stimulating factors by the endothelium. The secreted cytokines attract monocytes, which then adhere to endothelial cell adhesion molecules, transmigrate through the vascular wall and accumulate around proliferating collateral arteries. After infiltration of the perivascular tissue, the monocytes mature to macrophages, producing numerous cytokines and degrading enzymes, thereby creating the controlled inflammation necessary to remodel an arteriole into an artery.

The described scenario of adhering leukocytes, transendothelial migration and cell activation is to a large part similar to the processes occurring during inflammation, and the factors involved are often identical. Recent studies have shown that non-steroidal anti-inflammatory drugs (NSAID) inhibit leukocyte accumulation during inflammatory diseases. Although the mechanism of this anti-inflammatory action is not completely understood, the inhibition of the cyclooxygenases (COX) has been proven to play a crucial role. ASA is a potent irreversible inhibitor of both COX-1 and COX-2. In addition to its inhibitory effect on leukocyte migration, it further inhibits subsequent leukocyte activation.
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ASA also inhibits platelet aggregation. As inhibitors of platelet aggregation have been shown to reduce the follow-up mortality after cardiac events, ASA is the most prescribed drug in long-term treatment of patients with coronary heart disease. Novel compounds such as clopidogrel demonstrated a favorable safety-tolerability profile, with a significantly lower overall incidence of gastrointestinal hemorrhage, abnormal liver function, and indigestion but an increase in the rate of skin rash and diarrhea. Furthermore, clopidogrel is not associated with hematological side effects that limit the use of the ADP receptor antagonist ticlopidine. Since the anti-platelet effect of clopidogrel is not mediated via inhibition of cyclooxygenases, it therefore does not exhibit comparable anti-inflammatory action such as ASA.

Since arteriogenesis depends on monocyte migration and activation, we hypothesized that treatment with ASA potentially inhibits arteriogenesis after arterial occlusion, whereas other anticoagulative agents (e.g. clopidogrel) might not.

Our hypothesis was further based on the result of the CAPRIE-trial, in which an amplification of the absolute benefit of clopidogrel over ASA in patients with a history of symptomatic atherosclerotic disease was observed. As this observation might also be due to a preferential effect of clopidogrel on collateral artery growth, we applied ASA or clopidogrel to rabbits after femoral artery ligation and assessed the effects on the collateral circulation.

Material and Methods

This study was performed after securing appropriate institutional approvals. It conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). After ligation of the right femoral artery as previously described, 54 New Zealand White rabbits (NZWR) were randomly assigned to one of three groups. n=18 each. Group 1 consisted of animals receiving normal saline orally, whereas animals of group 2 received clopidogrel per os once daily in a concentration of 10 mg/kg body weight via gavage. Group 3 received ASA (acetylic salicylic acid) (10 mg/kg body weight/d) analogous to group 2. Oral administration of the substances showed to be easy and safe and was well tolerated by the animals. No animal suffered gangrene, necrosis or gross impairment of hind limb function after the initial operation. There was no observable difference in post-operative behavior or body weight between any group at any time-point.

During the observation period, blood samples were withdrawn from 6 animals per group for leukocyte activation assessment. Seven days after femoral artery ligatation, the animals were again anesthetized for either post-mortem angiography, hemodynamic measurements or preparation of histological samples.

Postmortem angiograms
After maximal vasodilatation with adenosine to exclude interference of neurogenic vascular tonus, legs were perfused with PBS in a warmed waterbath (37°C) for 1 minute at a pressure of 80 mmHg, followed by perfusion for 8 - 10 minutes at 80 mmHg with contrast medium based on bismuth and gelatin according to a formula developed by Fulton. Subsequently, the contrast medium was allowed to gel by placing the limbs on crushed ice for 45 minutes.
Collateral arteries were classified according to Longland \(^23\) into stem, midzone and reentry. Collateral vessels were marked after counting to make sure that no vessel was counted twice.

**Hemodynamic measurements**

Collateral conductance measurements were performed as previously described \(^24\). In short, a pump-driven shunt between left carotid artery and abdominal aorta was installed by cannulating both vessels. Pre-stenotic blood pressure was measured at the place of the aortic cannula insertion. Post-stenotic blood pressure was measured by cannulating the saphenous artery right above the ankle. The left femoral artery was occluded and cannulated for withdrawal of blood flow reference samples for subsequent calculation of the specific blood flows to each tissue sample. Using a roller-pump, six different flow respectively pressure levels were maintained under continuous flow and pressure monitoring. Maximal vasodilation was achieved by continuous adenosine infusion into the shunt system (1mg/kg body weight per minute). At each pump level, fluorescent microspheres (Molecular Probes, Leiden, The Netherlands) with a different label (scarlet, crimson, red, blue-green, orange and yellow-green) were injected. Tissue samples for the evaluation of collateral dependent flow were harvested, weighed and then further processed for flow cytometric quantification of microspheres per tissue sample. Flow was then calculated from the number of microspheres in the samples and plotted against the respective pressure levels (pressure gradient of pre-stenotic minus post-stenotic pressure). Collateral conductance as the incline in blood flow per increase of the pressure gradient equals the slope of the resulting curve of the six different pressure to flow relations.

**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. Directly after the withdrawal, whole blood was stained for CD-11a, CD-11b and CD-18 (all Serotec, UK) using specific mouse anti-rabbit antibodies. For identification of monocytes, blood samples were furthermore stained with a PE-conjugated anti CD-14 antibody (DAKO, Glostrup, Denmark). One group of samples was immediately analyzed via flow-cytometry (Epics XL, Beckman-Coulter)

The remaining blood samples were then divided into three smaller samples, from which the first one was left untreated and served as control. Samples two and three were stimulated with LPS (200ng/ml) and MCP-1 (200ng/ml) respectively. After addition of the factors, all samples were incubated at 37\(^\circ\) C in a 5% CO\(_2\) atmosphere for 2 hours. Subsequent to the incubation period, blood 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 vs. non-stimulated blood.

**Histological examinations of in-vivo cell migration**

Frozen tissue sections (5 \(\mu\)m) were cut of the specimens of each animal and stained for Ki-67 (mouse anti-rat Ki-67, Clone MIB-5, DAKO) to quantify the proliferative index of the media, stained with a specific antibody against alpha-smooth muscle
actin (SMC) (Sigma Chemical Company, St. Louis, MO). The proliferative index of each vessel was calculated as the percentage of Ki-67 positive cells of cells within the media of the growing collateral arteries. Ki-67 is a specific marker for proliferating cells and only growing collateral arteries are positively marked, whereas vascular smooth muscle cells from quiescent “normal” arteries, tissue feeding arteries, remain negative. The proliferative index of growing collateral arteries within the very early phase correlates with the rate of growth and thus the size of the vessels.

To examine and quantify the number of transmigrated leukocytes, the tissue sections were furthermore stained with a specific mouse anti-rabbit CD11b (Mouse anti-Rabbit CD11b, Clone 198. Serotec, Oxford, UK) antibody, staining granulocytes and monocytes/macrophages. Only cells undoubtedly showing a positive stain and an identifiable nucleus were counted. Additionally, monocytes/macrophages were identified by staining the sections with a mouse anti-human CD68 antibody (Monoclonal Mouse anti-Human CD68, Macrophage, Clone EBM11. DAKO, Glostrup, Denmark), which has been shown to cross-react with rabbit monocytes/macrophages in previous studies. Again, only positive marks around a nucleus were identified as macrophages and counted.

Statistical analysis
Results are expressed as mean ± SEM. Statistical analysis between treatment groups and control group were performed with SigmaStat (SPSS Inc., Chicago) using one way analysis of variance (ANOVA) and post-hoc Bonferroni correction.

Results

Post-mortem angiograms
Post-mortem angiograms performed subsequent to the observation and treatment period of seven days showed numerous collateral arteries spanning from the arteria circumflexa femoris and the arteria profunda femoris, thereby bypassing the stenosis caused by the ligation of the femoral artery.

However, there was no significant difference between any of the groups. Neither the number nor the diameter of angiographically detectable collateral arteries was measurably altered by the treatment regimen (solvent: 16.7 ± 1.2; clopidogrel: 17.3 ± 1.2; ASA: 16.2 ± 2.0) (Fig. 1).
Hemodynamic measurements
Collateral conductance without further treatment seven days after ligation showed a 5-fold increase as compared to acute occlusion of the femoral artery. Daily oral administration of clopidogrel at 10 mg/kg body weight did not affect collateral artery growth as detected by collateral conductance measurements (solvent: 50.7 ± 4.7 ml/min/100mmHg, clopidogrel: 49.5 ± 5.8 ml/min/100mmHg; p=NS). In contrast, oral treatment with ASA using the same dosage, led to a significant reduction of the collateral conductance as compared to untreated as well as to clopidogrel treated animals (ASA: 32.6 ± 3.9 ml/min/100mmHg; solvent vs. ASA p<0.05; clopidogrel vs. ASA p<0.05) (Fig. 2).
Leukocyte activation
Immediately after withdrawal of the blood samples, no significant difference in Mac-1 and LFA-1 expression on monocytes and granulocytes was detected between untreated, ASA treated and clopidogrel treated animals. However, the relative increase of Mac-1 (CD 11b) and LFA-1 (CD 11a) expression as markers of cellular activation was significantly reduced by a prior ASA treatment as compared to leukocytes from untreated animals. In contrast to the findings in ASA treated animals, clopidogrel treatment did not affect the efficacy of monocyte activation by MCP-1 (CD 11a expression: solvent: 1.27 ± 0.02; clopidogrel: 1.31 ± 0.04; ASA: 1.18 ± 0.03; p<0.05; CD 11b expression: solvent: 1.26 ± 0.04; clopidogrel: 1.29 ± 0.03; ASA: 1.22 ± 0.03, p=ns) and LPS (CD 11a expression: solvent 1.38 ± 0.04; clopidogrel: 1.39 ± 0.03; ASA: 1.15 ± 0.03; p<0.05) (CD 11b expression: solvent 1.50 ± 0.03; clopidogrel: 1.42 ± 0.03; ASA: 1.36 ± 0.04; p<0.05) (Fig. 3a-d).
Figure 3a

Figure 3b
Figure 3c

Figure 3d

Figure 3a-d: Flow cytometric analysis of monocyte activation under clopidogrel or ASA treatment. In-vivo treatment with ASA significantly reduced the ability of circulating monocytes to respond to stimuli with proper activation.

Histological examination of in-vivo cell migration
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. The amount of transmigrated monocytes/macrophages in ASA treated animals was significantly reduced compared to the aforementioned groups (solvent 26.96 ± 3.27; clopidogrel 22.87 ± 1.26; ASA 17.54 ± 0.42 CD68 positive cells mm²) (Fig. 4, Fig. 5a).
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 ASA significantly reduced leukocyte migration (solvent 33.06 ± 2.33, clopidogrel 29.57 ± 1.89, ASA 18.54 ± 1.26 CD11b positive cells/mm²) (Fig. 4, Fig. 5b).

**Figure 4:** Immunohistological staining for monocytes, macrophages, and proliferation. ASA treatment significantly reduced leukocyte migration and proliferation, clopidogrel treated animals showed comparable results as solvent treated animals.

![Immunohistological staining](image-url)
Figure 5a-b: Leukocyte migration into the perivascular space of growing collateral arteries. ASA treatment significantly inhibited leukocyte migration as compared to solvent or clopidogrel treatment.

**Proliferation indices**

Ki-67 staining showed growth and proliferation of arteriolar anastomoses to functional collateral arteries, whereas "normal" muscle feeding vessels remained negative. Qualitatively, collateral arteries from ASA treated animals showed a reduced number of Ki-67 positive cells (Fig. 4). Quantitative analysis confirmed this observation, as ASA treatment significantly reduced the proliferation index (Ki-67...
positive cells vs. negative cells) in the media of the vessels (solvent 23.10 ± 1.20; clopidogrel 23.53 ± 0.44; ASA 19.15 ± 0.46 % Ki-67 positive cells) (Fig 6).

Figure 6: Proliferation after solvent, clopidogrel or ASA treatment in growing collateral arteries. ASA application significantly reduced the rate of proliferation of the media of the vessels, confirming functional parameters.

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 formation. Numerous therapeutic options, invasive and conservative, have been established for the treatment of the increasing number of patients suffering from atherosclerosis. However, an important pharmaceutical component of almost every treatment regimen is the inhibition of platelet aggregation to ameliorate the rheologic properties of the blood and to prevent thrombotic occlusion of vessels. For this purpose, the most often prescribed drug is ASA. More recently, novel compounds (e.g. clopidogrel) have been developed to inhibit platelet aggregation, although the pharmacodynamic mechanisms are different. ASA irreversibly and unselectively blocks the cyclooxygenases (COX-1 and COX-2), whereas clopidogrel (or its metabolites) acts as a non-competitive antagonist of the P2Y₁₂ ADP-receptor on platelets. Since platelet inhibitors have become the standard medication for the treatment of patients with cardiovascular diseases, alone or in combination, this study focused on the role of ASA and clopidogrel on collateral artery growth (arteriogenesis). Moreover our hypothesis was supported by the observation of the CAPRIE-investigators, who described an relative risk reduction for stroke, myocardial infarction of death due to vascular causes of 8.7% under clopidogrel compared to ASA in more than 19000 patients with a history of symptomatic atherosclerotic disease.

In fact, bypassing collateral arteries may - upon critical stenosis of the main feeding artery - protect peripheral regions from critical ischemia and tissue
destruction. Hence the adaptive proliferation of collateral pathways (arteriogenesis) is an important natural rescue mechanism to compensate for arterial occlusion. In patients with cerebral arterial stenosis the degree of collateralization directly correlated with infarct volume and survival. Moreover, it becomes obvious, that clinical compounds used to chronically treat patients with vascular stenotic disease should not negatively affect adaptive arteriogenesis.

NSAID and their effect on vessel growth, in particular their influence on angiogenesis (i.e. formation of new capillary networks) have been subject of numerous studies. Although recent studies showed comparable anti-angiogenic properties of ASA and other NSAID in different models, the underlying mechanisms have not yet been clarified. However, arteriogenesis differs from angiogenesis: Collateral vessels are preexisting rather than newly built vessels. The substrates of arteriogenesis are arterioles which remodel into functional arteries rather than capillary sprouts. Furthermore, the growth of collateral arteries is not depending on ischemia, but mainly induced by increased hemodynamic shear forces.

To the best of our knowledge, this is the first study to report an inhibitory effect of ASA on arteriogenesis. The rabbit hindlimb model is well-established and widely used to examine the effect of different compounds on 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.

In our study collateral conductance (as the functional parameter of collateral artery growth) was significantly decreased by daily oral administration of ASA. Compared to placebo-treated control animals, conductance was reduced by ~40%. Quantitative immunohistochemistry for vascular proliferation (Ki-67) confirmed these hemodynamic results, as ASA treated animals showed significantly lower numbers of Ki-67 positive cells in the media of the growing collateral vessels seven days after femoral artery ligation.

As previously described, monocytes/macrophages are the key mediators of arteriogenesis, in particular during the very early stages. In ASA treated rabbits, the amount of CD 68 positive macrophages in the adventitial tissue of the growing collaterals was significantly reduced as compared to untreated and clopidogrel treated animals. As described above, the mechanisms underlying arteriogenesis are to a great part the same or similar to the ones responsible for inflammatory processes. Previous studies have shown that NSAID significantly inhibit leukocyte/macrophage adhesion to endothelium in vivo and in vitro, the first mandatory step of the arteriogenic response in early phases after acute arterial occlusion. Subsequently to the endothelial adhesion, leukocytes transmigrate into the perivascular tissue, where they produce several chemokines and growth factors, also inhibited by ASA as shown recently.

Monocyte transmigration is mediated by different cell adhesion molecules. Immunohistological studies showed, that within 24 hours after femoral artery ligation in the rabbit hind limb ICAM-1 and VCAM-1 are upregulated and expressed by the endothelium in growing collateral vessels. Leukocytes (e.g. monocytes) attach via interaction of the heterodimers CD18/CD11b (Mac-1) and CD18/CD11a (LFA-1) with ICAM-1 on the endothelium. Mac-1 is recognized to be an early marker of monocyte activation and is upregulated by different activating...
factors (e.g. LPS, MCP-1). The amount of CD11b on the surface of monocytes corresponds to its activation status. We therefore tested, whether monocytes from ASA treated animals show a comparable activation response to LPS and MCP-1 as monocytes from untreated or clopidogrel treated animals. FACS-analysis revealed that increase in CD11b expression following in-vitro cytokine stimulation of ASA treated monocytes was significantly lower than the activation potential of untreated monocytes. This might explain the decreased numbers of monocytes/macrophages in the peri-vascular tissue after ASA treatment. As a consequence, the deficit in growth factors released by macrophage leads to a reduced proliferation of the collateral vasculature, resulting in a reduced functionality of the collateral network; 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 upon ASA treatment might negatively affect progress of cardiovascular disease, compared to other compounds equal anti-thrombotic action, but without this unwanted side-effect.

In fact, in the CAPRIE study -the largest prospective evaluation of antiplatelet agents and the first study to include patients with all of the main clinical manifestations of atherothrombosis in a single clinical trial- an amplification of the absolute benefit of clopidogrel over ASA in patients with a history of symptomatic atherosclerosis was observed (especially concerning the effects in PAD patients).

**Conclusion**

In summary, this is the first study demonstrating a direct inhibitory effect of ASA on collateral artery growth. Newer platelet inhibitors, such as clopidogrel, acting via different mechanisms other than cyclooxygenase inhibition do not affect the natural arteriogenic response. The possible clinical relevance of this observation will be tested prospectively in the ongoing ART.NET-1 trial.
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