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Clopidogrel reduces the development of transplant arteriosclerosis

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Background: Transplant arteriosclerosis, the hallmark feature of chronic rejection, is still the major limiting factor for the long-term success of heart transplantation. Platelets have been implicated to play a role in the pathogenesis of this disease. Therefore the aim of this study was to investigate whether platelet inhibition alone has a positive effect on the development of transplant arteriosclerosis.

Methods: Fully major histocompatibility complex–mismatched C57BL/6 (H2^b) donor aortas were transplanted into CBA (H2^k) recipients, and mice received different doses (1, 10, and 20 mg/kg) of clopidogrel or control saline as a daily intraperitoneal injection for 30 days. Blood was analyzed on days 2, 7, 14, and 30 by using a platelet aggregation test (adenosine diphosphate) for effectiveness of the treatment. Grafts were analyzed by means of histology and morphometry on day 30 after transplantation.

Results: When mice were treated daily with 1 mg/kg clopidogrel in the absence of any other immunosuppression, transplant arteriosclerosis was significantly reduced compared with that seen in saline-treated control animals (intimal proliferation of 66% ± 9% [1 mg/kg clopidogrel] vs 77% ± 5% [control], n = 7, P ≤ .03). Daily application of 10 mg/kg and 20 mg/kg clopidogrel also significantly reduced the development of transplant arteriosclerosis compared with that seen in control animals (intimal proliferation of 61% ± 11% [10 mg/kg clopidogrel] vs 54% ± 10% [20 mg/kg clopidogrel] vs 77% ± 5% [control], n = 8, P ≤ .003). There was, however, no additional beneficial effect when compared with mice treated with 1 mg/kg clopidogrel (P = .06). Isografts did not show any signs of vascular lesions on day 30 after transplantation.

Conclusion: These results demonstrate that monotherapy with clopidogrel can effectively reduce the formation of transplant arteriosclerosis in a murine aortic allograft model.

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Transplant arteriosclerosis, the hallmark feature of chronic rejection, is still the leading cause of late mortality and limits the long-term success of heart transplantation.¹ It is characterized by a diffuse and progressive thickening of the arterial intima that affects minor, as well as major, coronary arteries of transplanted cardiac allografts.^{2,3} This results in sudden or chronic progressive ischemic damage to the transplanted heart, with subsequent organ failure. As the transplanted heart is denervated, the first clinical manifestations of transplant arteriosclerosis might be progressive heart failure, ventricular arrhythmia, or sudden cardiac death. Transplant arteriosclerosis is diffuse and frequently involves long segments of the affected arteries. In contrast to ordinary arteriosclerosis, which usually affects one part of the artery more severely than other parts and produces prominent lesions, transplant arteriosclerosis is characterized by concentric intimal thickening.^{2,3}

Abbreviations and Acronyms

ADP	= adenosine diphosphate
CD40L	= CD40 ligand
PDGF	= platelet-derived growth factor

Numerous pathogenetic mechanisms have been suggested to be involved in the development of transplant arteriosclerosis, including immune-mediated vascular injury, inflammation of the vascular endothelium, ischemia-reperfusion injury, cytomegalovirus infection, and metabolic risk factors.^{2,3} The role of platelets during the development of ordinary arteriosclerosis is meanwhile established,^{4,5} and interactions of platelets with the endothelium induce significant changes in the adhesive and chemotactic properties of endothelial cells that trigger monocyte adhesion and transmigration. This results in early inflammation during the process of arteriosclerosis within the vessel wall,^{6,7} and therefore recent interest has focused on the involvement of platelets in the pathogenesis of transplant arteriosclerosis. While comparing the results of intracoronary ultrasonographic examinations and platelet analyses from patients undergoing heart transplantation, Fateh-Moghadam and colleagues⁸ showed that platelet activation by increased expression of ligand-induced binding site 1 was strongly associated with the development and progression of transplant arteriosclerosis. In a similar study Hognestad and associates⁹ could demonstrate an enhanced expression of P-selectin, CD63, and soluble CD154 by platelets from patients undergoing heart transplantation severely affected by transplant arteriosclerosis.

Clopidogrel, a member of the thienopyridines, has become an important therapeutic agent for patients with coronary heart disease^{10,11} and has been shown to decrease the incidence of coronary artery stent thrombosis and to reduce myocardial infarction, stroke, and vascular death within these patients.¹¹ It inhibits platelet activation by blocking an adenosine diphosphate (ADP) receptor on platelets, recently designated P2Y₁₂.^{12,13} Clopidogrel is inactive *in vitro* and requires hepatic metabolism for production of its active metabolite, which has also been identified recently.¹⁴ It seems unlikely that the clinical benefit of this drug can be adequately explained by its ability to decrease ADP-induced platelet aggregation alone, suggesting that other not yet defined mechanisms, such as a platelet-mediated inflammatory reaction, might play a role.¹³

This study tested the hypothesis as to whether treatment with clopidogrel (Plavix, Sanofi-Synthelabo, Berlin, Germany) alone results in a significant reduction of transplant arteriosclerosis. Mouse abdominal aortic allografts were used as the experimental model because they have been shown to represent vascular lesions similar to those ob-

served in human coronary arteries that are affected by transplant arteriosclerosis and therefore allow a precise analysis of the composition of the vascular lesions.¹⁵

Materials and Methods**Animals**

C57BL/6 (H2^b) and CBA.J (H2^k) mice were originally purchased from Charles River (Sulzbach, Germany). C57BL/6 (H2^b) mice were used as donors, and CBA.J (H2^k) mice were used as recipients of the aortic allografts. All mice used in this study were aged between 6 and 12 weeks at the time of experimental use and were bred and maintained at the animal facility of the Department of Experimental Surgery at the University of Erlangen-Nuernberg under specific pathogen-free conditions and treated in accordance with institutional and state guidelines.

Treatment Protocol

Clopidogrel (Plavix) was obtained from the local hospital pharmacy, and 75-mg tablets were dissolved in 0.9% saline under sterile conditions. This solution was then diluted appropriately in the following concentrations: group 1, 1 mg/kg clopidogrel equivalent of a human daily dose; group 2, 10 mg/kg clopidogrel; and group 3, 20 mg/kg clopidogrel. Daily treatment with clopidogrel was started immediately after transplantation for 30 days. Because dissolved clopidogrel is unstable,¹⁴ the clopidogrel solution was freshly prepared every day and injected intraperitoneally immediately after preparation. The overall injection volume was 0.5 mL for each treatment group.

Platelet Aggregation

For *ex vivo* platelet aggregation, blood was collected in 3.2% citrate. Approximately 0.5 mL of blood could be obtained from each mouse, and samples were immediately processed after blood drawing. Platelet aggregation was evaluated by means of optical aggregometry in citrated blood samples at 37°C by using a 2-channel Chronolog aggregometer Elvi Logos, Milan, Italy. Because mice have slightly more platelets compared with human subjects, for technical reasons, the amount of citrate within the test tubes was increased to 500 μ L of citrate (500 μ L citrate/500 μ L blood) for *ex vivo* platelet aggregation. Therefore in our setting even untreated control animals showed a relative reduction of platelet aggregation to about 50%. However, this did not affect absolute differences seen in platelet aggregation between the experimental groups. Platelet-rich and platelet-poor plasma was prepared from citrated whole blood by means of centrifugation (100g for 10 minutes). The final platelet count was adjusted to an average of 2.5×10^5 platelets/mL with autologous plasma. Twenty microliters of ADP Sigma, St Louis, Mo; final concentration, 2×10^{-4} mol/L, respectively was added to induce platelet activation, and aggregation was recorded for at least 10 minutes. Maximal aggregation was mostly seen around 5 minutes and was used as a measurement of aggregation.

Abdominal Aortic Transplantation

The procedure was performed with a modified technique initially described by Koulack and coworkers.¹⁶ In brief, the donor thoracic aorta was isolated, resected, and transferred to the recipient animal.

The recipient aorta was clamped and then transected with sharp microvascular scissors. A proximal end-to-end anastomosis was performed. The aortic graft was then repositioned, and the anastomosis was continued with single interrupted sutures.

Analysis of the Aortic Graft

Aortic grafts were removed after achievement of anesthesia on days 14 and 30 after transplantation. Grafts were perfused with saline and were flash frozen in OCT medium (Tissue-Tek, Sakura, Netherlands) in liquid nitrogen for morphometric analysis of 5- μ m cryostat sections. A minimum of 10 transverse sections was analyzed from each graft.

Morphometry

Five sections from each graft harvested at days 14 and 30 were stained with Elastin-van Gieson and analyzed by 2 independent examiners (S.A. and S.M.E.) blinded to the experimental conditions at an original magnification of 200 \times using a conventional light microscope. A digitized image of each section was captured, and the areas within the lumen and the internal and external elastic lamina were circumscribed manually and measured as previously described.¹⁷ All image analyses were carried out on a color display monitor with ANALYSIS Image Analysis software (Olympus, Hamburg, Germany).

Statistical Analysis

Results are given as the mean per group \pm standard deviation, which was derived from the mean per graft. The data were analyzed by using a 2-tailed unpaired Student *t* test.

Results

Platelet Aggregation Was Effectively Inhibited After Clopidogrel Administration

Ex vivo blood samples were harvested on days 2, 7, 14, 21, and 30 to evaluate the effect of clopidogrel on platelet function and to ensure sufficient platelet aggregation inhibition within the CBA/J recipient mice. Aggregation was determined by means of light transmittance aggregometry in response to ADP (2×10^{-4} mol/L, Figure 1). Blood drawn from recipient mice treated with 1, 10, and 20 mg/kg clopidogrel showed significantly reduced platelet aggregation on day 2 ($26\% \pm 7\%$ [1 mg/kg clopidogrel, $n = 5$, $P \leq .05$] vs $12\% \pm 4\%$ [10 mg/kg clopidogrel, $n = 5$, $P \leq .03$] vs $9\% \pm 3\%$ [20 mg/kg clopidogrel, $n = 5$, $P \leq .01$] vs $45\% \pm 5\%$ [control]) and day 7 ($16\% \pm 4\%$ [1 mg/kg clopidogrel, $n = 5$, $P \leq .03$] vs $9\% \pm 2\%$ [10 mg/kg clopidogrel, $n = 5$, $P \leq .01$] vs $4\% \pm 1\%$ [20 mg/kg clopidogrel, $n = 5$, $P \leq .001$] vs $48\% \pm 4\%$ [control]) after transplantation (Figure 1). Application of 10 mg/kg and 20 mg/kg clopidogrel also significantly reduced platelet aggregation compared with 1 mg/kg clopidogrel on day 2 ($12\% \pm 4\%$ [10 mg/kg clopidogrel, $n = 5$, $P \leq .05$] vs $9\% \pm 3\%$ [20 mg/kg clopidogrel, $n = 5$, $P \leq .03$] vs $26\% \pm 7\%$ [1 mg/kg clopidogrel]) and day 7 ($9\% \pm 2\%$ [10 mg/kg clopidogrel, $n = 5$, $P \leq .05$] vs $4\% \pm 1\%$ [20 mg/kg clopidogrel, $n =$

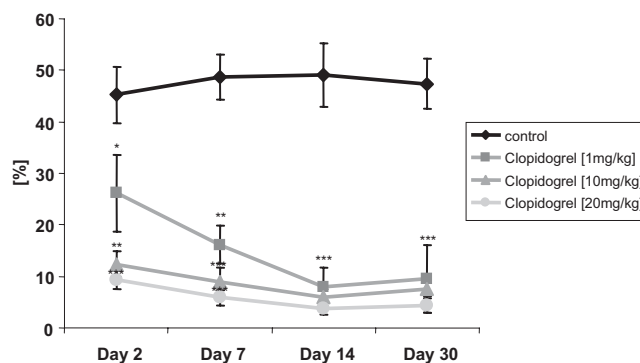


Figure 1. Effect of clopidogrel on adenosine diphosphate (ADP)-induced platelet aggregation in mice after transplantation. Platelet aggregation was studied after stimulation with ADP (20 μ mol/L) by means of light transmittance aggregometry. * $P < .05$ versus untreated control animals, ** $P < .03$ versus untreated control animals, * $P < .01$ versus untreated control animals ($n = 5$ animals per group).**

5, $P \leq .03$] vs $16\% \pm 4\%$ [1 mg/kg clopidogrel]; Figure 1). From day 14 onward, platelet aggregation in all groups was significantly inhibited compared with that seen in untreated control animals, and there were no differences between 1, 10, and 20 mg/kg clopidogrel (Figure 1). Blood from untreated control animals showed unimpaired platelet function throughout all time points (Figure 1).

Clopidogrel Significantly Reduced the Development of Transplant Arteriosclerosis

A total of 58 transplantations were performed for this study, with an overall complication rate of 6.8%, the most frequent complication being thrombosis of the aortic graft ($n = 3$) and one late recipient death caused by other courses. Aortic allografts (C57BL/6 [H-2b]) from recipients (CBA/J [H-2^k]) were analyzed 30 days after transplantation, the time point at which distinctive changes of transplant arteriosclerosis are most evident.¹⁸ When recipient mice were treated daily with 1 mg/kg clopidogrel in the absence of any other immunosuppression, transplant arteriosclerosis was significantly reduced compared with that seen in untreated control animals (intimal proliferation of $66\% \pm 9\%$ [1 mg/kg clopidogrel] vs $77\% \pm 5\%$ [control], $n = 8$, $P \leq .03$; Figures 2, B and C, and 3). Daily application of 10 mg/kg and 20 mg/kg clopidogrel also significantly reduced the development of transplant arteriosclerosis compared with that seen in untreated control animals (intimal proliferation of $61\% \pm 11\%$ [10 mg/kg clopidogrel, $n = 8$, $P \leq .003$] vs $54\% \pm 10\%$ [20 mg/kg clopidogrel, $n = 8$, $P \leq .001$] vs $77\% \pm 5\%$ [control]; Figures 2, D and E, and 3). However, there was no additional beneficial effect when compared with mice treated with 1 mg/kg clopidogrel ($P = .06$,

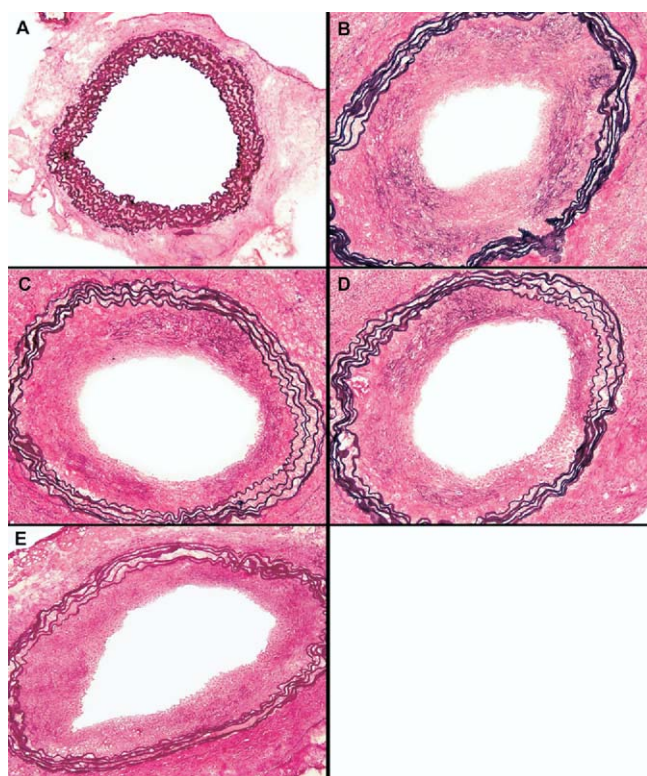


Figure 2. Histopathologic evaluation of the morphology of untreated fully allogeneic C57BL/6 aortic grafts implanted into CBA recipients showed high levels of intimal proliferation (B), whereas C57BL/6 aortic grafts of recipients treated with 1 mg/kg clopidogrel (C), 10 mg/kg clopidogrel (D), and 20 mg/kg clopidogrel (E) showed significantly reduced intimal proliferation 30 days after transplantation. Syngeneic control animals did not show any vascular lesions (A). The tissue was snap-frozen, and sections were stained with Miller’s Elastin–van Gieson stain. (Original magnification 200×.)

Figure 3). Syngeneic control grafts (C57BL/6 [H2^b] into C57BL/6 [H2^b] recipients) did not show any signs of transplant arteriosclerosis at 30 days after transplantation, indicating that nonimmunologic mechanisms alone were not sufficient to initiate the development of transplant arteriosclerosis in this model (Figure 2, A). None of the recipient mice showed any kind of hemorrhage or major postoperative bleeding.

Discussion

The development of transplant arteriosclerosis in cardiac allografts is a multifactorial process, with macrophages, T cells, proinflammatory cytokines, adhesion molecules, growth factors, and alloantibodies implicated in both the initiation and progression of this chronic inflammatory process.^{3,19} It is well established nowadays that platelets play an important

role in thrombus formation and atherogenesis, but several studies suggest that these cells might also play an important role in inflammation.^{6,20}

This study demonstrates that (1) treatment with different doses of clopidogrel significantly inhibited platelet aggregation in a murine aortic allograft model, (2) that monotherapy with clopidogrel significantly reduced the development of transplant arteriosclerosis in the absence of any additional immunosuppression, and (3) that there was no significant beneficial effect when the daily clopidogrel dose was increased. The level of reduction of intimal proliferation by clopidogrel was as effective as anti-CD8⁺ T-cell depletion.¹⁷ These findings imply that circulating platelets in recipients with aortic transplants might contribute to the development of transplant arteriosclerosis.

Three different treatment groups (1, 10, and 20 mg/kg) were examined to find the most effective dose of clopidogrel for the prevention of transplant arteriosclerosis. Administration of 1 mg/kg clopidogrel, the equivalent of a human daily dose, already showed a strong platelet aggregation inhibitory effect and resulted in a significant reduction of transplant arteriosclerosis. Kinetic analysis revealed that higher doses of clopidogrel caused a more rapid inhibition of platelet aggregation but failed to prove an additional therapeutic effect. These results imply that reduction of the development of transplant arteriosclerosis by clopidogrel might not be entirely due to its ability to decrease ADP-induced platelet aggregation, suggesting that additional immunomodulatory effects, such as a platelet-mediated inflammatory reaction, might play a role.¹³

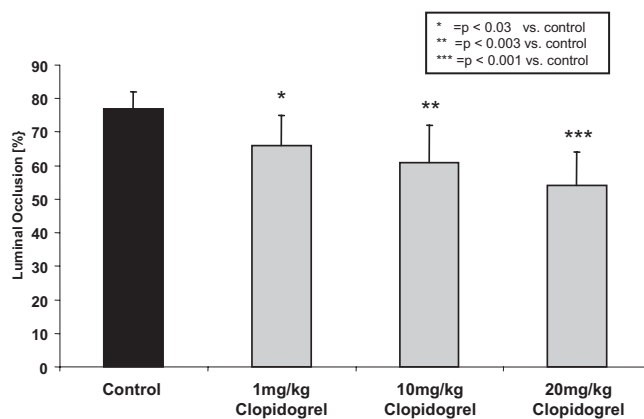


Figure 3. For the morphometric analysis of the degree of intimal thickening, Miller’s Elastin–van-Gieson–stained sections were used. Areas within the lumen and the internal and external elastic lamina were circumscribed manually and measured. From these measurements, a quotient for the thickness of the intima (Q_{int}) was calculated. Q_{int} indicates the relative thickness (in percentage) of the intima. Five measurements from different areas of each aortic graft were obtained for this analysis. (n = 8 animals per group, P values as indicated in the diagram).

TX

Activated platelets are able to release a number of inflammatory compounds from their granules, such as P-selectin²¹ and platelet-derived growth factor (PDGF),²² that can activate monocytes, granulocytes, and endothelial cells, all contributing to a systemic inflammatory response in various pathologic conditions, such as transplant arteriosclerosis.^{3,19} In addition, platelets can induce enhanced surface expression of adhesion receptors, such as intercellular adhesion molecule 1²³ and E-selectin²⁴ on endothelial cells that mediate adhesion and transmigration of leukocytes, an essential step during the development of transplant arteriosclerosis.²⁵ Furthermore, activated platelets contain high levels of intracellular soluble CD40 ligand (CD40L).⁶ Most of these molecules have been shown to be involved in the pathogenesis of transplant arteriosclerosis.

Experimental data from carotid artery allografts have demonstrated that intracellular adhesion molecule 1 plays a critical role through modulation of leukocyte adhesion and infiltration into the vessel wall.²⁶ During chronic rejection in a murine heterotopic cardiac allograft model, the intensity of arterial intimal thickening was significantly correlated with the intensity of P-selectin expression on endothelial cells, and therefore a crucial role for P-selectin in the pathogenesis of transplant arteriosclerosis by augmenting immune-mediated injury was concluded.²⁷ In addition, Lemstrom and associates²⁸ showed that suppression of PDGF expression inhibited the development of transplant arteriosclerosis through regulation of cell migration and proliferation. Finally, the importance of CD40L-CD40 interaction during the development of transplant arteriosclerosis has been highlighted in numerous experimental studies.^{29,30}

In a prospective clinical study Fateh-Moghadam and colleagues⁸ found that transplant arteriosclerosis in patients receiving heart transplants was associated with enhanced fibrinogen receptor activation (ligand-induced binding site 1) and P-selectin surface expression of the circulating platelets. These authors concluded that increased activation of circulating platelets in these patients might contribute to the development and progression of transplant arteriosclerosis. Similar results were obtained by Hognestad and coworkers,⁹ demonstrating enhanced expression of P-Selectin and soluble CD40L by analyzing platelet activation in long-term survivors of heart transplantation. They could also show that heart transplant recipients were characterized by a persistent immune activation even several years after transplantation, suggesting that enhanced platelet activation might have contributed to this inappropriate immune activation after heart transplantation.

Clopidogrel inhibits platelet activation by blocking the P2Y₁₂ ADP receptor on platelets.¹² Analysis of the relationship between P-selectin expression and PDGF secretion in human platelets revealed that treatment with clopidogrel resulted in downregulation of P-selectin expression and re-

duced PDGF secretion.³¹ When patients with anti-thy1 glomerulonephritis were treated with clopidogrel, they showed significantly limited extracellular matrix deposition, as well as transforming growth factor β_1 expression,³² both pathologic mechanisms involved in the formation of transplant arteriosclerosis.^{30,33} Recently, it was also shown that administration of clopidogrel reduced P-selectin and CD40L expression in an experimental rabbit ischemic coronary artery model.³⁴ Experiments to further clarify and understand the underlying pathologic mechanisms of the beneficial effect of clopidogrel on the development of transplant arteriosclerosis are currently ongoing in our laboratory. However, we already think at this stage that effective anti-platelet strategies might be a reasonable addition to the clinical therapy to improve the long-term outcome of heart transplantation.

In conclusion, we have shown that monotherapy with clopidogrel can effectively reduce the formation of transplant arteriosclerosis in a murine aortic allograft model. Furthermore, clopidogrel is a readily available and widely used drug with an established clinical safety profile. These findings therefore have important clinical implications because patients who are predisposed to or have transplant arteriosclerosis after cardiac transplantation might substantially benefit from treatment with this drug.

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