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Microvascular integrity plays an important role for graft survival after experimental skin transplantation

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ABSTRACT

Background: Every transplanted organ relies on a reliable and sound vascular system. Therefore, our study focused on the investigation if platelet inhibition alone or combined with mTOR-inhibition has a beneficial effect on the microvascular integrity in allogeneic murine skin grafts.
Methods: Skin transplantation was performed from fully MHC-mismatched C57BL/6 (H-2^b) donors to CBA/J (H-2^k) recipient mice. Skin allograft recipients were assigned to several experimental groups and either treated with clopidogrel alone, everolimus alone or a combination of both. Graft survival was evaluated and transplants were harvested after 8 days and analyzed for CD31 and C4d by immunohistochemistry.
Results: Untreated allografts showed a reduced amount of CD31 on postoperative day 8 as well as an increase in C4d compared to isografts. All treated animals showed a significant improvement regarding CD31 [1577.7 ± 200.4 (clopidogrel)/1702.8 ± 151.1 (clopidogrel + everolimus) vs. 479.7 ± 184.2 (control), $n = 8$, $p < 0.05$] and C4d [420.9 ± 70.9 (clopidogrel)/324.5 ± 77.3 (clopidogrel + everolimus) vs. 772.4 ± 159.7 (control), $n = 8$, $p < 0.05$]. In addition, skin grafts of animals treated with clopidogrel and everolimus survived significantly longer compared to untreated controls [19.2 ± 4.2 d vs. 12.8 ± 2.4 d, $n = 10$, $p < 0.05$].
Conclusion: In this study we could show that clopidogrel alone and in combination with everolimus substantially improved microvascular integrity and resulted in increased survival time of skin grafts.

1. Introduction

Despite dramatic donor organ shortage, cardiac transplantation still remains the gold standard for the treatment of end-stage heart failure patients. Progress in the development of immunosuppressive therapy has decreased the incidence of acute rejection episodes, but the development of transplant coronary arteriosclerosis continues to limit the survival of heart transplant recipients and is still the leading cause of late mortality after heart transplantation [1]. Different pathogenic 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].

As interactions of platelets with the endothelium induce significant changes in the adhesive and chemotactic properties of endothelial cells that are able to trigger monocyte adhesion and transmigration resulting in an inflammatory reaction throughout the vessel wall [3,4], our recent interest has focused on the involvement of platelets in the pathogenesis of transplant arteriosclerosis. Here we could recently show that monotherapy with clopidogrel effectively reduced the formation of transplant arteriosclerosis in a fully allogeneic murine aortic allograft model [5], even when it was administered in a delayed fashion [6]. Clopidogrel, a member of the thienopyridines, has become an important therapeutic agent for patients with coronary heart disease [7,8] and clopidogrel therapy has been shown to decrease the incidence of coronary artery stent thrombosis and to reduce myocardial infarction, stroke and vascular death within these patients [8]. It inhibits platelet activation by blocking the adenosine diphosphate (ADP) receptor P2Y12 on platelets [9,10].

As monotherapy with clopidogrel seemed to have no influence on the T-cell infiltration of the allogeneic aortic grafts a combination of a T-cell modulating agent such as a calcineurin antagonist (cyclosporine) and mTOR inhibitor (everolimus) was investigated in the following set of experiments. Only treatment with clopidogrel and everolimus resulted in a striking reduction of transplant arteriosclerosis, whereas application of cyclosporine and clopidogrel showed no additive effect [11]. This was also demonstrated in an orthotopic tracheal transplant model, where we could show that platelet inhibition with clopidogrel in reduced levels of T-cell and macrophage infiltration is associated

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with diminished tracheal obliteration and lower expression levels of inflammatory cytokines and adhesion molecules. Concomitant application of an mTOR inhibitor had an additive effect and further reduced the formation of obliterative airway disease (OAD) [12]. In this context it has been shown that untreated orthotopic tracheal allografts undergo a decrease of vascular perfusion associated with a loss of endothelial cells in the early days after transplantation whereas isografts and immunosuppressed allografts were preserved [13]. Having shown an inhibitory effect of clopidogrel in mice on platelet activation [5] we speculated at this stage that beside immunosuppressive effects, targeting platelets by clopidogrel may also mitigate the process of OAD by improving allograft perfusion and therefore reduce intragraft tissue hypoxia as platelet inhibition is well known to have a beneficial effect on hemodynamics in particular within the microvasculature.

2. Objective

Therefore, the aim of the current study was to investigate if platelet inhibition alone or combined with mTOR-inhibition has a beneficial effect on the microvascular integrity in a murine skin transplantation model. This particular experimental model was chosen, as graft survival of skin transplants mainly depends on the blood supply provided through the microvessels within the skin and therefore if our hypothesis is correct, preservation of microvascular integrity would result in improved skin graft survival.

3. Material and methods

3.1. Transplants

All mice were treated and housed according to protocols approved by the animal care and use committee of the government (Regierung Mittelfranken, AZ: 54–2532.1–46/12) in the Franz-Pentzold-Zentrum (Erlangen, Germany). C57BL/6 (H-2^b) and CBA/J (H-2^k) mice aged between 8 and 12 weeks were received from Janvier (Le Genest Saint Isle, France). Skin-transplantation was performed similar to the technique initially described by Billingham and Medawar [14]. Briefly, the skin from the tail of a euthanized C57BL/6 mouse was removed by applying a circular incision at the base and pulling it off. Gender-matched CBA/J as recipient was anesthetized with isoflurane and prepared for transplantation by shaving the hair of the left dorsal chest. A small piece of adhesive tape was used as a frame and the inside this frame about 1 cm^2 of the epidermis and dermis was removed leaving the muscles intact. The grafts were fitted into the frame and bandaged for 8 days.

3.2. Treatment protocol

All drugs were administered by intraperitoneal injection and the recipient mice were treated according to the following protocol. The animals were treated either with clopidogrel alone (C: 20 mg/kg), everolimus alone (E: 0.05 mg/kg) or a combination of both drugs (EC: 20 mg/kg clopidogrel and 0.05 mg/kg everolimus). There were also two untreated groups as controls, untreated allografts as positive control (Po) and a group with isografts as negative control (Ne). This treatment pattern was already introduced by our group [12,15]. Since clopidogrel is not stable, we daily prepared the solutions for the intraperitoneal injections. Clopidogrel (Plavix©; Sanofi-Aventis, Berlin, Germany) and everolimus (Certican©; Novartis, München, Germany) was obtained from the hospital pharmacy and further processed and diluted to reach desired concentrations of the respective drug.

3.3. Immunohistology

For immunohistochemical analysis animals were sacrificed on postoperative day 8, harvested skin-transplants were covered in Tissue-Tek® (Sakura, Alphen aan den Rijn, The Netherlands) and flash frozen in liquid nitrogen. The tissue was then cut into 4 μm slices, air-dried and fixed for 10 min in acetone.

Slides were rehydrated and pre-incubated in staining buffer (0.1 Tris, pH 7.5 and 0.1% Tween 20) for 5 min. Afterwards sections were incubated with the primary antibodies at room temperature for 60 min. The slides were then washed and treated for 60 min with secondary antibodies. Visualization was reached with Liquid Permanent Red (Dako Deutschland GmbH; Hamburg, Germany). After 10 min the slides were washed with ultrapure water, stained with hematoxylin and washed again. The final step was the sealing with Vecta Mount AQ (Vector Laboratories, Inc.; Burlingame, CA, U.S.A). A digitized image of the respective stained section was analyzed with cellSens Dimension (Olympus, Germany). A predefined frame was applied on the image and the stained area inside the frame was measured (Fig. 1). The frame covered the whole area from the epidermis to the hypodermis in all samples. We analyzed five sections of each specimen and averaged the results before comparison.

The following antibodies and conjugates were used: rabbit anti-mouse CD31 and rabbit anti-mouse C4d (Abcam plc; Cambridge, UK) as primary antibodies, and goat anti-rabbit IgG-AP (Invitrogen, Life Technologies GmbH; Darmstadt, Germany) as secondary antibody.

3.4. Graft-survival

Graft viability was analyzed by daily observation and photo documentation of the transplanted skin. During the rejection process the blood supply into the graft is progressively restricted, which can be observed as an increasing area of necrosis within the graft. Transplants were classified as vivid if the necrotic part was less than 75%.

3.5. Platelet aggregation

In order to ensure an effective inhibition of the platelet aggregation throughout the whole experiment, we investigated the effect of the applied drugs on the platelets with a Multiplate© analyzer (Roche, Switzerland). In contrast to our previously published experiments [12, 15] blood could be used without further processing instead of preparing platelet rich plasma (PRP) first. 500 μl of blood was collected from the treated mice and immediately mixed with 100 μl of a prepared hirudin-solution (acquired from Monovette®, Sarstedt AG, Germany), so the final concentration of hirudin in the sample amounted to 0.0675 mg/ml. The mixed blood was than analyzed using multiple electrode aggregometry after stimulation with 0.02 mml/l ADP as described by Tóth et al. [16]. The read out was the area under the aggregation curve measured in “U”.

3.6. Statistical methods

The data in the graphs are presented as means ± standard deviation. The comparison of two groups of the histological analysis was performed by a Student's T-Test with an adaption according to Bonferroni–Holm. For the analysis of the graft-survival we used a Log-Rank-Test with a Chi-Quadrat-Test for post-hoc testing.

4. Results

4.1. Monitoring platelet aggregation after treatment with clopidogrel alone and in combination with everolimus

To ensure effective platelet inhibition, blood was collected from treated CBA/J (H-2^k) mice as well as an untreated control group on days 3 and 8. Platelet aggregation was analyzed by multiple electrode aggregometry. Both groups treated with 20 mg/kg clopidogrel showed a significant impaired aggregation in comparison with untreated controls on day 3 (11.7 ± 2.3 U [clopidogrel] vs. 34.0 ± 13.5 U [untreated