Repetitive transplantation of different cell types sequentially improves heart function after infarction

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Abstract

Cell-based therapy is considered a novel and potentially new strategy in regenerative medicine. But the efficacy of cell-based therapy has been limited by the poor survival of the transplanted cells in an ischaemic environment. The goal of the present study is to present a possibility to increase survival of the transplanted cardiomyocytes, by increasing the vascularization of the infarcted area. First, we injected endothelial progenitor cells (EPCs) to augment the vascular density in infarcted areas and to improve the benefit of a subsequent Tx of foetal cardiomyocytes. Serial echocardiography indeed showed significant improvement of the left ventricular function after application of EPC and a significant additive improvement after Tx of foetal cardiomyocytes. In contrast, repetitive EPC transplantation as a control group did not show an additional improvement after the second transplantation. Histologically, cells could be readily detected after Tx by BrdU-staining for EPC and by carboxy-fluorescein diacetate succinimidyl ester (CFSE)-staining for foetal cardiomyocytes. Staining for CD31 revealed a significant increase in vessel density in the infarction area compared with medium controls, possibly contributing to the benefit of transplanted foetal cardiomyocytes. Notably, a significant increase in the number of apoptotic cells was observed in cell-transplanted hearts accompanied by an increase in proliferation, collagen content and neutrophil infiltration, suggesting an active remodelling concomitant with sustained inflammatory processes. In conclusion, repetitive Tx of different cell types after myocardial infarction in rat hearts significantly improved left ventricular function and could represent a feasible option to enhance the benefit of cell therapy.

Keywords: cell-based therapy • myocardial infarction • repetitive cell transplantation

Introduction

Recent years have seen a considerable rise in studies employing transplantation of various cell types in different models of myocardial injury [1]. The ultimate goal of cell-based cardiac repair is regeneration of healthy, functionally integrated, myocardial tissue. However, it remains unclear which cell type is most effective, which cell preparation and transfer technique is optimally suited and, last but not least, which mechanisms are primarily responsible for improving heart function. Several studies have postulated that neovascularization is the main reason for improved heart function [2–5]. We could previously demonstrate that transplantation of endothelial progenitor cells (EPCs) [6] or endothelial cells [7] improved heart function after transplantation in border zones of myocardial infarction (MI). Evidence for a significantly higher amount of vessels in infarcted areas pointed towards neovascularization to be responsible for a better contractile function. More recently, we could show that an inflammatory reaction to
transplanted cells seemed to further support the scar remodelling and paracrine regeneration of damaged myocardium [8]. But also the transplantation of foetal cardiomyocytes improved myocardial function in a rat model [5]. This effect seemed to be due to intercellular connection of transplanted cells to host cells and an additive contractile effect, which could in part account for preserved myocardial function. But independent of the cell-type, the efficacy of cell-based therapy has been limited by the poor survival of the transplanted cells in an ischaemic environment.

The goal of the present study is to present a possibility to increase survival of the transplanted cells, in our case cardiomyocytes, by increasing the vascularization of the infarcted area. Premaratne et al. could demonstrate that a repetitive transplantation of skeletal myoblasts is possible and can lead to a significant improvement of LV function, which can secure a sufficient graft cell mass without tumour-like overgrowth [9]. The EPC transplantation should improve neovascularization and reconstitute vascular structures in damaged myocardial areas. That should increase the survival chances and the benefit of transplanted foetal myocardial cells in damaged myocardium.

This is the first study that attempts to elucidate for the first time whether repetitive cell transplantation of two different cell types augments myocardial function in a synergic way and can represent a promising and accessible strategy to optimize cardiac repair in the future.

Materials and methods

Generation of myocardial infarctions

Forty-eight female adult Sprague-Dawley rats (same age and same weight 200–250 g) were intubated under general anaesthesia (1 ml/kg ketamine and 10 mg/kg xylasine, intraperitoneal) and positive pressure ventilation was maintained (room air supplemented with oxygen) using a rodent respirator. Hearts were exposed through a 2-cm left thoracotomy and MI was induced by suture occlusion of the left anterior descending artery between the left atrium and the right pulmonary outflow tract (7/0 polypropylene; Ethicon, Norderstedt, Germany). The muscle layer and skin incision were closed with a silk suture. Animal experiments were approved by local authorities and complied with German animal protection law.

Identification of the cells and analysis of the infarction area

For cell identification, slides (5 µm) were stained with anti-BrdU kit (Zymed). CSFE-stained cardiomyocytes appear in red after staining with an anti-FITC (Sigma-Aldrich, St. Louis, MO, USA) antibody and alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA, USA). Universal quick kit and alkaline phosphatase substrate kit (Vector Laboratories) were used with anti-CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for vessel identification. Only the ring-like structure positive for CD31 in infarcted areas was counted in six different fields in three different sections from the same heart only and expressed as vessels/mm². Accustain trichrome stain (Masson; Sigma-Aldrich) was used to determine collagen content of infarcted regions. The stained areas were measured by computer-assisted planimetry (Diskus software, Hilgers, Königswinter, Germany). Macrophages and Neutrophiles were stained with alpha-naphthyl acetate esterase (Sigma-Aldrich) and naphthol as-d chloroacetate (Sigma-Aldrich), respectively. The nuclei undergoing apoptosis were stained with MEBSTAIN apoptosis kit II (MBL, Woburn, MA, USA). To evidence proliferation, the sections were incubated with BrdU (Zymed, Vienna, Austria) as described by the manufacturer. We characterized in detail these cells in our previous works [5, 6, 8], so we do not provide in this study additional data about the markers analysis for the isolated EPCs and foetal cardiomyocytes.

Cell transplantation

Transplantation was performed 4 weeks and 12 weeks after MI. The rats were anaesthetized and the hearts were exposed through thoracotomy as described above. The areas for injection were chosen by visual identification based on surface scarring and wall motion akinesis. Cells were transplanted into marginal zones of the MI by syringe injection (for 1-min injection time) at three distinct but adjacent sites. Animals were divided into three groups, 10–19 animals each: the control group received only culture medium (RPMI), the second group received EPCs and foetal myocardial cells (1 × 10⁶), the third group received twice EPC (1 × 10⁶). We chose these cell types and time points of transplantation based on our recently published studies [5, 6]. After injection, the puncture holes were closed by suture, which served as a marker for the area of transplantation at follow-up thoracotomy. As immunosuppressive treatment, we administrated Cyclosporin A orally in both groups, starting 1 week prior to the first transplantation at a dose of 50 mg/kg [5].

Echocardiography

Four weeks after MI (i.e. prior to transplantation), 2 months after first transplantation and 1 month after second transplantation, rats were anaesthetized and two-dimensional and M-mode measurements were performed with a SONOS 7500 (Philips GmbH, Hamburg, Germany) with a 15-MHz linear phased-array probe. The animals were placed in the supine or lateral position and excessive pressure on the thorax was avoided. Parasternal long-axis and short-axis views were obtained, ensuring that the mitral and aortic valves and apex were well visualized. Area fraction and wall area were determined by planimetry of end-diastolic and systolic volumes in parasternal short axis. Measurements of LV end-diastolic and end-systolic dimensions were obtained in M-mode from more than three beats and fractional shortening (FS) was calculated as FS (%) = (LVIdd – LVIDs)/LVIDdd × 100, where LVID is LV internal dimension, s is systole and d is diastole.
stained for Ki-67 (DAKO, Hamburg, Germany), followed by an anti-rat-FITC antibody [10]. The stained cells were counted only in infarcted areas and express as cells/mm². Double staining were performed for BrdU and CD31 to assess the incorporation of the transplanted cells in the newly formed vessels, as well as for Ki-67 and CD31 or ED-1 (Macrophages marker; Serotec, Oxford, UK) to prove the cells type with proliferative activity.

Data analysis

Data represent mean ± SD. Statistical analysis was performed with Prism 4 software (Graph Pad) using unpaired Student’s t-test or one-way ANOVA followed by Newman-Keuls test. Differences with P < 0.05 were considered significant.

Results

Tracking of transplanted cells

Endothelial progenitor cells were transplanted four weeks after ligation of the left coronary artery. After additional 8 weeks foetal cardiomyocytes (n = 19) or a repetitive EPC transplantation (n = 10) as well as medium (n = 19) were injected into infarcted myocardium and adjacent border zones. Transplanted cells were labelled using BrdU (EPCs) and CFSE (foetal cardiomyocytes or repetitive transplanted EPC) to allow for further tracking. We were able to detect BrdU-labelled EPCs, of which some are incorporated in capillary-like structures (Fig. 1A), as shown also by double staining with CD31 (Fig. 1B), as well as CFSE-labelled foetal myocardial cells surrounded by fibrous tissue in infarcted areas of host myocardium after two transplantations (Fig. 1A).

Remodelling and inflammation in infarcted areas

TUNEL staining revealed significantly more apoptotic cells in infarcted areas after cell transplantation in cardiomyocyte-transplanted group (661.5 ± 39.0 TUNEL⁻ cells/mm² versus control 441.9 ± 113.5 TUNEL⁻ cells/mm²; P < 0.05, Fig. 2A). This result differs from our previous findings showing less apoptosis after single transplantation of EPCs [6], microspheres or fibroblasts [8], indicating a more marked remodelling in the present model. This notion is further supported by increased proliferation in the infarcted area of the cardiomyocyte-transplanted group (113.1 ± 20.98 Ki-67⁺ cells/mm² in cell transplanted group versus control 41.7 ± 11.4 Ki-67⁺ cells/mm², P < 0.05, Fig. 2B). Contrary, the EPC-transplanted group showed no differences in TUNEL⁻ cells (430.0 ± 41.1 TUNEL⁻ cells/mm², Fig. 2A), as well as in proliferation (42.3 ± 5.9 Ki-67⁺ cells/mm², Fig. 2B).
compared with control. We found the presence of Ki-67 mostly in endothelial cells (Fig. 2C), but we found also some macrophages with proliferating activity (Fig. 2D).

Additional evidence for increased remodelling could be provided, namely collagen content, angiogenesis and inflammatory cell content. Analysis of collagen content by specific staining revealed a significant increase in the cardiomyocyte-transplanted hearts (41.8 ± 2.3% of infarcted area), as well as in the EPC-transplanted group (43.7 ± 1.9% of infarcted area versus 35.1 ± 1.6% of infarcted area in controls, \( P < 0.05 \), Fig. 3A). The infarct...
size before injection did not differ between the groups (data not shown). To investigate effects on angiogenesis, vascular density was assessed by staining and quantification of CD31-positive vessels in the area of infarction. Both groups with cell injection resulted in a significantly increased number of vessels after Tx as compared to medium treated control hearts (2495 ± 150 vessels/mm² in cardiomyocyte-transplanted group, 2563 ± 116.5 vessels/mm² in EPC-transplanted group versus 1567 ± 151 vessels/mm² in controls, *P < 0.006, Fig. 3B). Notably, the number of vessels determined after two transplantations was considerably higher than after a single transplantation, as described previously [6, 7]. Furthermore, as an indicator of inflammation, we assessed monocyte/neutrophil cell infiltration in all groups by staining for specific/unspecific esterase. Infiltration of neutrophils in the infarcted areas was significantly more marked in cardiomyocytes-transplanted hearts (12.5 ± 2.6 cells/mm²) and EPC-transplanted hearts (13.83 ± 2.2 cells/mm² versus 5.6 ± 0.8 cells/mm² in medium controls, *P < 0.05; Fig. 3C), while no difference was found in the numbers of monocytes/macrophages.

**Cardiac function**

To assess cardiac function, we performed 2D echocardiography before and after MI, the first and second transplantation, and at the end of the experiment. Four weeks after MI, reduced FS was observed in all groups. Remarkably, after transplantation of both cell types, echocardiographic analysis revealed a significant improvement of FS after transplantation of EPCs and an additional significant improvement after the second transplantation of foetal cardiomyocytes (FS: after MI 30.3 ± 7.1% versus control 30.4 ± 9.1%; after first EPC-Tx 36.8 ± 6.2% versus control 30.6 ± 7%; *P = 0.007; after second foetal cardiomyocyte-Tx 41.2 ± 5.2% versus control 31.1 ± 10.4%, *P = 0.008; Fig. 4). In the EPC-transplanted group, a significant improvement in heart function was observed after the first transplantation, the second transplantation failed to add a sequential benefit, compared with cardiomyocyte-transplanted group (FS: after MI 28.1 ± 6.5% versus control 30.4 ± 9.1%; after first EPC-Tx 37.8 ± 7.2% versus control 30.6 ± 7%, *P = 0.007; after second EPC-Tx 35.6 ± 7.8% versus control 31.1 ± 10.4%, *P = 0.008; Fig. 4).
Discussion

Various studies have made an effort to elucidate and scrutinize the mechanisms, which were responsible for beneficial effects in infarcted myocardium following cell-based therapy [4, 7, 8, 11, 12]. Repairing vascular integrity, stimulating neovascularization [6], attenuated loss of myocardial function [5], modulation of inflammatory processes [8] or paracrine effects [11, 13–16] might be in part responsible for cardiac protection. In the same way, genetically altered cells or cells preconditioned with growth factors have shown an additional impact on improvement of myocardial function [17–19].

The results of different studies lead to the conclusion that attenuation of myocardial dysfunction after cell transplantation seems to be a combination of different mechanisms. Different cell types were suggested to exert different mechanisms in improving myocardial function. Whereas the main mechanism of transplanted endothelial cells or EPCs may be improved neovascularization, through a better nutrition of infarcted regions and a partially reconstituted vascular network, the main beneficial effect of transplanted myocardial cells seems to be an additional reservoir of contractile cells. However, paracrine effects of both cell types have also been suggested as important mechanisms. The goal of the present study was to combine these different mechanisms by sequentially transplanting EPCs and foetal cardiomyocytes.

Recently, we demonstrated that transplantation of microspheres (uniform polystyrene, 10 μm diameter) into infarcted myocardial areas was able to improve myocardial function compared to medium injected control hearts [8]. Therefore, it was tempting to speculate that inflammation triggered by transplantation potentially contributes to and modifies remodelling processes. Similar to transplanted microspheres, transplantation of EPCs had a beneficial effect on myocardial function. Mechanisms of improved myocardial function appeared to be attributable to augmented neovascularization, as suggested by several other studies dealing with transplantation of endothelial cells. Indeed, our results showed significantly increased neovascularization, as evident by vessel density, which was even more profound than previously observed with a single transplantation of EPCs alone [6]. On the other hand, two times of EPC transplantation did not augment the vessel density compared to a second transplantation of foetal cardiomyocytes and did not lead to an additional improvement of myocardial function after a second injection. It has been further speculated that transplanted cells were able to acquire or support functional properties of resident cells, such as contractility of myocytes [5]. We could indeed show improved contractile function after transplantation of foetal cardiomyocytes [5]. But to reverse left ventricular remodelling and to improve cardiac function, a large number of cardiomyocytes is needed, which can represent a high risk of tissue overgrowth or massive cell death. Indeed, Reinecke and Murry have demonstrated a risk of tissue overgrowth that sometimes distorts the ventricular contour, when myoblasts are transplanted as a bolus [20]. Based on these findings, our hypothesis was that the EPC transplantation should improve neovascularization and reconstitute vascular structures in damaged myocardial areas. This improved "preconditioning" should increase the survival chances and the benefit of transplanted foetal myocardial cells in damaged myocardium.

Indeed, our results show that repetitive cell transplantation, even after three successive open-chest operations, is feasible and echocardiographic analysis and isolated heart studies according to Langendorff revealed a significant improvement of myocardial function after infarction compared to controls without cell transplantation. FS improved after first cell injection in the transplantation group, whereas no change of myocardial function is detectable in the control group. In contrast, despite the initial significant improvement of heart function after the first transplantation, repetitive EPC-transplantation failed to add a significantly benefit compared with cardiomyocyte-transplanted animals. This finding is surprising, since Premaratne et al. showed that a repetitive transplantation of a smaller volume of the same cell type can be more effective as a single injection of a larger volume of myoblasts. He showed that a bolus injection of myoblasts may lead to ischaemia in the centre of the area of transplanted cells, which is the case also in cardiomyocyte transplantation. In contrast, repeated transplantation of a smaller volume of cells may have protected the myoblasts from ischaemia-induced cell death [9].

Fig. 4 Echocardiographic results. Fractional shortening in percentage after transplantation revealed significant differences towards improved left ventricular function after transplantation of EPCs and an additional significant improvement after transplantation of foetal cardiomyocytes, which is not present in EPC-twice treated group. *P < 0.05 versus medium, §P < 0.05 versus after MI in the same group. £P < 0.05 versus after 1st Tx in the same group.
But knowing the effect of the single cell type transplantation, we
do not exclude that the mechanisms of the second transplantation
are more comprehensive as we analysed and presented. Despite
the efficiency of repetitive myoblast transplantation, repetitive EPC
transplantation did not present a real benefit, but is able to support
and improve the effect of the foetal cardiomyocyte transplantation.
Therefore, this strategy should be considered to increase the clinical
potential of cell therapy.

It has been suggested in previous studies that beneficial effects of
cell transplantation are partially caused by reduced numbers of
apoptotic cells in infarcted regions [6, 8]. In the present study we
dected significantly more apoptotic cells and increased proliferation
after second foetal cardiomyocyte transplantation. Therefore, we
assumed that repetitive transplantation of different cell types caused
a prolongation of the scar remodelling, explaining the improvement
in the heart function. We did not observe this effect after repetitive
EPC transplantation. Surprisingly, repetitive EPC transplantation
leads to the same increase in collagen content and angiogenesis as
the cardiomyocyte group, but no additional improvement in heart
function. That could be only explained by different paracrine effects
of the EPCs and foetal cardiomyocytes, and this demonstrates the complexity of the processes and necessity for more profound analysis of molecular events preceding cell therapy.

Besides, inflammatory response should play the major role in
starting these remodelling processes. As we showed in our previous
study [8], the inflammation induced by injection of unlikely candidates such as fibroblasts and even microspheres 1 month after MI contributed to remodelling and improved myocardial function. Furthermore, standard pharmacologic therapy following MI including statin and ACE inhibitor therapy is known to modulate inflammatory processes. Recently, Ciulla et al. observed similar mechanisms in improving heart function by standard ACE inhibitor treatment ACE in experimental MI compared with cell-administration [21]. In the present study we observed an increased inflammatory response and we supposed that could be an important mechanism in initiating the remodelling of the infarction scar. As we showed previously, single cell transplantation induces mostly an increase in monocyte/macrophage recruitment, but not neutrophils [6–8]. In this study, the sequential transplantation of EPC and foetal cardiomyocytes as well as the repetitive transplantation of EPC did not affect monocyte/macrophage recruitment, but induced an increased neutrophil infiltration. It seems that despite the effect on inflammation differed between the two different cell types, the sequential transplantation might act synergic and amplified the response.

Beyond the limitations, our study tried to open new sights of the
cell-therapy, which have not yet been explored. Our present experimental study has demonstrated that sequential transplantation of different cell types further improves cardiac function concurrent with reverse LV remodelling compared with a single cardiomyocytes transplantation. But of course, a better understanding of these multifaceted inflammatory interactions warrants a complex and comprehensive further investigation. Also further studies in large animal models are required to improve the reproducibility, to monitor long-term effects and to facilitate a clinical approach.

In conclusion, our study demonstrates that repetitive trans-
plantation of different cells is feasible and is associated with a sequential improved myocardial function. The mechanisms of altered myocardial function by cell transplantation are still unclear and need further investigation. At this time point, improvement of myocardial function appears to be a combination of different mechanisms, additional contractility by transplanted contractile cells, augmented neovascularization, paracrine effects and modulation of inflammatory processes. The main impact is likely to depend on the selection of cell types, mode of application, timing of application and combination of these factors.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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