Intramyocardial microdepot injection increases the efficacy of skeletal myoblast transplantation\textsuperscript{☆}

Harald C. Ott\textsuperscript{a,}*, Ruth Kroess\textsuperscript{b}, Nikolaos Bonaros\textsuperscript{b}, Rainer Marksteiner\textsuperscript{c}, Eva Margreiter\textsuperscript{c}, Thomas Schachner\textsuperscript{b}, Guenther Lauffer\textsuperscript{b}, Steffen Hering\textsuperscript{d}

\textsuperscript{a}Center for Cardiovascular Repair, University of Minnesota, 312 Church Street, 55455 Minnesota, MN, USA
\textsuperscript{b}Department of Cardiac Surgery, University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria
\textsuperscript{c}Department of Biochemical Pharmacology, University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria
\textsuperscript{d}Department of Pharmacology and Toxicology, University of Vienna, Althanstrasse 14, 1090 Wien, Austria

Received 13 September 2004; received in revised form 5 January 2005; accepted 20 January 2005; Available online 7 April 2005

Abstract

Objective: Recent progress in the field of cellular cardiomyoplasty has opened new prospects for the treatment of ischemic heart disease and currently moves from bench to bedside. The aim of the present study was to develop a novel cell delivery technique, reducing target tissue damage and improving cell dispersion and engraftment. Methods: In 30 male Fischer F344 rats an infarction of the left ventricle was generated by ligation of the left anterior descendent artery. Seven days after infarction, either 15 microdepositions of 10 \( \mu \)l myoblast cell suspension (microdepot group) or culture medium (control group) were injected into the infarcted region using an automatic pressure injection device, or three depositions of 50 \( \mu \)l myoblast cell suspension (macropot group) were injected using the standard surgical technique. Echocardiography was performed in all rats before and 6 weeks after cell injection. In all groups the perioperative mortality was below 20%. Six weeks after cell transplantation, a significant improvement of ejection fraction was seen in both myoblast treated groups compared to controls. In all groups the perioperative mortality was below 20%. Six weeks after cell transplantation, a significant improvement of ejection fraction was seen in both myoblast treated groups compared to controls (macropot, microdepot, control; \( 53.7 \pm 11.9\), \( 70.7 \pm 2.0\), \( 39.1 \pm 6.4\), \( P = 0.026, P < 0.001 \)). The microdepot group showed a more decent improvement than the macropot group (70.7 ± 2.0 vs. 53.7 ± 11.9, \( P = 0.013 \)). In both treated groups, grafted myoblasts differentiated into multinucleated myotubes within host myocardium, however, the engraftment pattern was different and angiogenesis was enhanced in the microdepot group. Conclusions: Intramyocardial multisite pressure injection allows the safe and reliable transplantation of several myoblast microdeposits into an infarcted myocardium and improves the efficacy of myoblast transplantation compared to the standard technique.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Stem cell; Heart; Myocardial infarction; Cellular cardiomyoplasty; Myoblast; Delivery

1. Introduction

Intramyocardial cell transplantation opened new perspectives for the treatment of ischemic heart disease [1]. In a number of myocardial injury models, different cell lines have proven the potential to regenerate viable tissue after being transplanted into the infarcted heart [1,2-5]. Among those, autologous skeletal myoblasts directly transplanted into damaged myocardium have improved myocardial performance in vitro and in vivo [1,7]. These convincing preclinical data lead to the first application in a clinical setting by Menasche et al. reported in 2001 [6] and the conductance of phase I clinical trials [7,8].

The majority of preclinical studies focused on different cell lines and their particular efficacy in several models of ischemic heart disease. However, apart from the development of new endoventricular approaches only little work has been published concerning different surgical delivery techniques and further application related issues [9,10]. In rodent models, skeletal myoblasts were usually injected in one to three sites in and around the infarcted myocardium. Corresponding to these early experiences, in the recently published clinical trials, skeletal myoblasts were directly injected in several sites in and around the scar tissue using standard syringes (25, 26 and 27 gauge) [7,8]. However, the achieved rate of engraftment seems everything else than satisfying. Histological data of one trial even estimate the total myoblast survival to be below 1% [8]. The low engraftment rate is discussed to be partially caused by an insufficient delivery method, allowing cellular back flow from the injection site and providing only little myoblast dispersion in the target tissue. These data raise the question...
for the development and evaluation of new cell delivery methods.

In the present study, we, therefore, propose a novel technique of surgical intramyocardial cell delivery, improving cell dispersion and engraftment without an increase in target tissue damage. By the use of an automated time gated pump, the fast application of several microdeposits of a certain volume at a defined pressure on the beating heart is possible. The simultaneous use of a modified syringe reduces the risk of transmural puncture with consecutive intraventricular injection and bleeding.

2. Materials and Methods

2.1. Isolation and Expansion of Skeletal Myoblasts

Primary skeletal myoblasts were isolated from a male F344 rat (6 weeks of age). Hind limb muscles (0.5–1.0 g) were dissected free from connective tissues, and minced into pieces of approximately 1 mm³. Muscle samples were enzymatically dissociated according to the cell dispersion technique described by Blau and Webster [11]. Before intramyocardial injection, cells were labelled using a fluorescent cell linker kit (PKH26-GL, Sigma-Aldrich Co., Vienna, Austria) following the instructions of the manufacturer as previously described [15].

2.2. Myocardial Infarction and Cell Transplantation

Myocardial infarction was induced following a standardized protocol: 30 male F344 Fischer rats were anaesthetised (Ketamine 0.1 mg/100 g bw and xylazine 0.02 mg/100 g bw intraperitoneally), placed in a supine position on a temperature controlled plate (37°C) and tracheally ventilated with a small animal Harvard respirator (Harvard Apparatus Rodent Ventilator Mod. 40–1003, Harvard Apparatus, Inc. Millis, MA). The heart was exposed through a 2 cm left lateral thoracotomy and the left coronary artery was ligated with a Prolene® 7/0 suture under the distal portion of the left atrial appendix. Correct position of the ligation was assessed by colour change of the ischemic area, regional wall motion and electrocardiographic (ECG) recording. After haemostasis was achieved, the muscle layer and skin incision was closed with Vicryl® 3/0 running sutures after drainage of the left thoracic cavity with a 16 G silicon tube. All animals were monitored for 4 h postoperatively.

One week after myocardial infarction, all animals underwent a second thoracotomy following the same protocol as above described. The heart was exposed via the same intercostal space as in the first operation and the infarcted area of the lateral left ventricular wall was clearly identified. Animals were now divided into three subgroups: the microdepot group, the macrodepot group and the control group. In the microdepot group 10³ skeletal myoblasts were injected into 15 microdeposets of 10 µl each in the centre and border zone of myocardial infarction. Injections were performed using a modified 24 G needle (Becton Dickinson, Helsingborg, Sweden) covered with a rubber tube allowing only a 1.5 mm puncture to avoid intracavitary injection. The needle was mounted on a modified 1 ml syringe that was connected via a polyethylene tube (outer diameter, 2.0 mm; inner diameter, 1.0 mm; Becton Dickinson, Helsingborg, Sweden) to a time gated pneumatic pump (PV 830, Pneumatic PicoPump, World Precision Instruments, Sarasota, FL, USA). The picopump was adjusted to a pressure of 30 psi at a gated impulse duration of 500–700 ms. Under these conditions a volume of 10 µl was injected intramyocardially with each pressure impulse. The surgeon triggered these pressure impulses by pushing a foot pedal after inserting the needle tip into the target region. In a separate experiment myoblast survival following this procedure (shear force in needle and syringe in vitro) was 95 ± 1.8%. Cell viability was assessed using a Guava ViaCount™ assay (Guava Technologies PCA 96, Guava, Hayward, CA). After accurate hemostasis the chest was closed and drained according to the above described technique. In the control group 15 depts of 10 µl empty culture medium (DMEM 1885-023, Gibco) were injected following the same protocol as in the microdepot group. In the macrodepot group the rethoracotomy was performed as described above. However, instead of creating 15 microdeposets, the same amount of cells as in the microdepot group (10³ myoblasts) was injected into three macrodeposets of 50 µl in the center and border zone of the myocardial infarction. In contrast to the other groups, the 24 G needle was connected to a standard 1 ml syringe and injections were performed manually. Myoblast survival following this procedure (shear force in needle and syringe in vitro) was 96 ± 1.2%. After closure of the chest all animals were monitored for 4 h and underwent the same postoperative routine.

2.3. Functional Assessment

Left ventricular function was assessed by two-dimensional echocardiography 1 week after myocardial infarction (at baseline, before cell transplantation) and 6 weeks after cell transplantation. Under general anaesthesia with ketamine (0.1 mg/100 g bw) the chest was shaved and a layer of acoustic coupling gel was applied. Two-dimensional and M-mode measurements were performed with a commercially available 15 MHz linear-array transducer system (AcuNav, Acuson Corp., Mountain View, CA). Parasternal long axis views were recorded and left ventricular dimensions and volumes were calculated according to standard formulas. All measurements were performed by two experienced investigators who were blinded to the treatment group.

2.4. Histology and Immune Histochemistry

Within 3 days after follow up echocardiography all animals were sacrificed with an overdose of ketamine and xylazine and hearts were harvested and cryopreserved for further histological analysis. The ventricles were cross-sectioned into three sections. From each section 8 µm slides were prepared using a cryostat and standard histological studies were performed with haematoxylin and eosin staining. The transplanted myoblasts were identified by fluorescence. For determination of microvascular density a von Willebrand Factor staining was performed. Cryosections were mounted on glass slides, rinsed
in PBS and fixed in 100% methanol at −20 °C for 5 min. All further incubations were carried out at room temperature and all rinses and dilutions were performed with a blocking solution consisting of 2% BSA and 0.5% Triton-X-100 in PBS. An initial blocking step was performed with this solution for 30 min. An antibody to von Willebrand Factor (F3520, Sigma) with a dilution of 1:200 was applied for 1 h. After rinsing a peroxidase-conjugated anti-rabbit IgG (A0545, Sigma) with a working dilution of 1:500 was applied for 30 min. The sections were rinsed and stained with AEC chromogen (3-Amino-9-Ethylcarbazole in N,N-dimethylformamide; IMMH5, Sigma). Endogenous peroxidase was quenched with 3% hydrogen peroxide. The number of capillaries was counted in the scar tissue of all three animal groups using a standard light microscope at a 400× magnification. Ten high-power fields in each scar were randomly selected, and the number of capillaries in each was averaged and expressed as the number of capillary vessels per high power field (0.2 mm²). Myoblast graft size and myotube count was measured in five randomly selected areas within each infarction scar of myoblast treated animals. Graft area was calculated using Image-Pro (Image-Pro plus 4.5.1.29 for Windows, Media Cybernetics, Inc.). The number of capillaries was counted, averaged and expressed as the number of capillary vessels per high power field.

2.5. Data analysis

Statistical analysis was performed using SPSS forwindows. In the following, data are expressed as mean ± standard deviation. Comparisons of continuous variables among animal groups were studied by a one way ANOVA. A standard deviation. Comparisons of continuous variables between cell injection, there was no significant difference in left ventricular enddiastolic diameter (LVEDD, control, macrodepot, microdepot; 6.8 ± 1.0, 5.8 ± 0.7, 7.0 ± 1.9, P = 0.184) among the different study groups. At follow up, the microdepot group showed a significantly smaller LVEDD compared to the control and the macrodepot group (6.5 ± 0.7 vs. 7.9 ± 0.5, P = 0.019 and 6.5 ± 0.7 vs. 8.0 ± 0.9, P = 0.014) (Fig. 1B). Concerning the LVEDD the macrodepot group did not differ from controls (8.0 ± 0.9 vs. 7.9 ± 0.5, P = 1.000).

2.6. Animal care

This study was approved by the Animal Care Commission of the University of Innsbruck and by the Ministry of Science, Republic of Austria. Care of animals was in accordance with the ‘Guide for the Care and use of Laboratory Animals’ (NIH publication 85–123, revised 1985).

3. Results

3.1. Functional assessment

Major results of functional assessment are summarized in Fig. 1. At baseline, 1 week after myocardial infarction but before cell injection, there was no significant difference in the assessed echocardiographic parameters. Thus, left ventricular ejection fraction (LVEF) was 53.1 ± 3.2 in control animals, 51.5 ± 15.9 in the group receiving macrodepos and 51.4 ± 7.7 in the group receiving microdepos (P = 0.427). The follow up echocardiography 6 weeks after cell transplantation revealed a significant improvement of ejection fraction in both myoblast groups compared to the corresponding values of the control group (in the macrodepot group 53.7 ± 11.9 vs. 39.1 ± 6.4, P = 0.026 and in the microdepot group 70.7 ± 2.0 vs. 39.1 ± 6.4, P < 0.001).

In contrast, a significant decrease in left ventricular function was observed in the control group compared to values of the baseline echocardiography (53.1 ± 3.2 vs. 39.1 ± 6.4, P = 0.027). Additionally, the microdepot group showed a more decent improvement in ejection fraction than the macrodepot group (70.7 ± 2.0 vs. 53.7 ± 11.9, P = 0.013) (Fig. 1A). At baseline echocardiography, there was no significant difference in left ventricular enddiastolic diameter (LVEDD, control, macrodepot, microdepot; 6.8 ± 1.0, 5.8 ± 0.7, 7.0 ± 1.9, P = 0.184) among the different study groups. At follow up, the microdepot group showed a significantly smaller LVEDD compared to the control and the macrodepot group (6.5 ± 0.7 vs. 7.9 ± 0.5, P = 0.019 and 6.5 ± 0.7 vs. 8.0 ± 0.9, P = 0.014) (Fig. 1B). Concerning the LVEDD the macrodepot group did not differ from controls (8.0 ± 0.9 vs. 7.9 ± 0.5, P = 1.000).

3.2. Histology and immune histochemistry

Representative histological slides of transplanted myoblast depots in the infarcted region of the left ventricular wall 6 weeks after cell transplantation are shown in Fig. 2. Groups of PKH labelled skeletal myoblast depots could be identified locally in the infarcted myocardium of all transplanted rat hearts. Corresponding to the different delivery techniques, myoblasts in the macrodepot group were found to be engrafted in single depots (Fig. 2A and B) whereas myoblasts in the microdepot group were distributed to several smaller regions in the infarcted free wall of the left ventricle (Fig. 2C and D). Examination of graft areas under fluorescence microscopy indicated that the size of particular myoblast depots was larger in the macrodepot group than in the microdepot group (0.319 ± 0.110 mm² vs. 0.092 ± 0.045 mm², P < 0.001). However, the area of myocardial infarction covered by myoblast grafts did not differ as cells were dispersed over multiple grafts in the microdepot group (0.319 ± 0.110 mm² vs. 0.360 ± 0.129 mm²).
Looking at those sections under light microscopy, the number of successfully engrafted multinucleated myotubes was larger in the microdepot group than in the macrodepot group. In the microdepot group differentiated myotubes were found to form from the border to the core zone of cell depots (Fig. 3A and B). In the macrodepot group a less organized pattern was observed, myotubes only formed in the border zone whereas necrosis and scar formation was observed in the core zone of cell depots (Fig. 3 C and D). Angiogenesis, as defined by capillary number, was found in the area surrounding grafted skeletal myoblasts in both myoblast treated groups (23.5 ± 6.6 capillaries per high-power field in the macrodepot group, 42.4 ± 14.5 in the microdepot group, \( P < 0.015 \) and \( P < 0.000 \) vs. the control group), but obvious angiogenesis was not found in the control group (11.7 ± 8.8 capillaries per high power field). Further, enhanced angiogenesis with a larger number of mature capillaries was observed in the microdepot group compared to the macrodepot group (23.5 ± 6.6 capillaries per high power field in the macrodepot group vs. 42.4 ± 14.5 in the microdepot group, \( P = 0.001 \)). Representative histological slides of von Willebrand stained infarcted myocardium are shown in Fig. 4.

### 4. Discussion

This study demonstrates that the pressure injection of autologous skeletal myoblasts in multiple microdepos may lead to a more distinct improvement in left ventricular function and an enhanced angiogenesis compared to the currently used cell transplantation technique, without increasing the risk of procedure related complications. The improved left ventricular function is related to an increase in myotube formation and differentiation within the injected cell depots. We speculate that these findings may result from an improvement in myoblast-scar interactions due to smaller depot diameters, an increased depot surface/volume ratio and therefore an increased contact area between myoblasts and host tissue (cumulative surface assuming the formation of ball-shaped depots: \( 3 \times \frac{5.9}{1.977} = 33.84 \text{ cm}^2 \) in the macrodepot group vs. \( 15 \times \frac{22.56}{33.84} = \text{cm}^2 \) in the microdepot group). This thesis is underlined by the fact that especially in the early stage after transplantation grafted cells are subjected to various pathological processes caused by environmental stress, such as ischemic and mechanical injury [12]. These stress factors are known to result in both necrosis and apoptosis of grafted myoblasts [1,13]. With the creation of microdepos, the increase of cell dispersion and the decrease of depot diameter may allow a better supply of transplanted cells.
myoblasts with oxygen, nutritional and environmental factors and therefore increase the survival and further differentiation of grafted cells. In the later phase of cell engraftment the increased cumulative graft surface and the smaller diffusion length within myoblast microdepots may enforce potential paracrine effects and may lead to an improved integration of differentiated myotubes within the scar matrix, thereby increasing any potential mechanical impact on left ventricular function [14]. The difference in left ventricular end diastolic diameter between animals of the microdepot group and animals of the macrodepot and control group indicates a beneficial effect on post infarction remodelling, possibly due to the above mentioned mechanisms.

In summary, the use of intramyocardial multisite pressure injection allows the safe and reliable transplantation of several microdepots into infarcted myocardium and may improve the efficacy of myoblast transplantation compared to the currently used open chest technique. This clinically relevant new surgical approach could be of importance for the treatment of patients with ischemic heart disease.

Acknowledgement

This work was supported by the FWF grant 15527 S. H.

References


Appendix A. Conference discussion

Dr M. Siepe (Freiburg, Germany): I was wondering what your control group received. Did they receive injections? And how many injections did they receive?

Dr Ott: That’s a very good question because needle injections have been discussed to cause an improvement in the left ventricular function or at least an increase in angiogenesis. To take this potential side-effect into account the control group received cell-free media in 15 injection sites, so the control group received microdepos of cell-free media.

Dr A. Wechsler (Philadelphia, Pennsylvania, USA): It’s interesting. You didn’t inject any control group with the larger dose just to test the hypothesis that that injection might induce injury and it looked better when you had cells in the 50 microliter injection.

Dr Ott: That’s true and we will do that. During the procedure of cell injection, you have the impression of inducing edema and a sort of acute stunning of the left ventricle. This effect seems to be worse with larger volumes.