Role of balloon occlusion for mononuclear bone marrow cell deposition after intracoronary injection in pigs with reperfused myocardial infarction

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Aims
In clinical studies on cell therapy for acute myocardial infarction (MI), cells are usually applied by intracoronary infusion with balloon (IC/B). To test the utility of balloon occlusion, mononuclear bone marrow cell (MNC) retention after intracoronary infusion without balloon (IC/noB) was compared with IC/B and intramyocardial (IM) injection.

Methods and results
Four hours after LAD ligation in male pigs, reperfusion was allowed (confirmed by coronary angiography). Five days later, $1 \times 10^8$ autologous $^{111}$Inium-labelled MNC were injected IC/noB ($n = 4$), IC/B ($n = 4$), or IM ($n = 4$). At 1 h the fraction of injected MNC that was detected in the heart was $4.1 \pm 1.1\%$ after IC/noB injection, $6.1 \pm 2.5\%$ after IC/B injection ($P = 0.19$), and $20.7 \pm 2.3\%$ after IM injection ($P < 0.001$ vs. IC/noB and IC/B). At 24 h it was $3.0 \pm 0.6\%$ (IC/noB), $3.3 \pm 0.5\%$ (IC/B, $P = 0.43$), and $15.0 \pm 3.1\%$ (IM, $P < 0.001$ vs. IC/noB and IC/B). Dynamic scintigrams during each of four consecutive IC/B injections showed a rapid $19.6 \pm 8.0\%$ cell loss during balloon inflation (no-flow period, phase 1) and a rapid $36.6 \pm 17.8\%$ cell loss after balloon deflation (re-flow period, phase 2). After each of four consecutive IC/noB injections the peak cell deposit was lower, followed by one phase of rapid cell loss ($30.9 \pm 11.0\%$ after 6 min). After IM injection only a slow linear cell loss was observed ($9.7\%$ per h). In histology, PKH-67 labelled cells only rarely had passed the endothelial barrier after 24 h after IC injection, while they were exclusively found in the interstitium after IM injection.

Conclusion
The observation of a similar cell persistence after IC injections with and without balloon occlusion suggests that the balloon procedures currently applied in clinical studies are not necessary for cell deposit. If longer term persistence of cells plays a role for the clinical benefit of cardiac cell therapy, IM injection may be superior to IC applications.

Keywords
Myocardial infarction • Cell therapy • Bone marrow cells • Mononuclear cells • Stem cells • Heart • Intracoronary • Intramyocardial • Pig • Organ distribution • Balloon
**Introduction**

Bone marrow cell transfer has been under investigation for the treatment of myocardial infarction (MI) and myocardial failure for several years. Although there is conflicting data, many experimental and clinical studies that used bone marrow cells for the treatment of MI showed beneficial results on cardiac function, perfusion, and remodelling.\(^1\)\(^-\)\(^^4\) In those studies, mononuclear bone marrow cells (MNC) are widely applied, their heterogeneity notwithstanding. Two major application techniques are used, i.e. intracoronary injection with balloon (IC/B)\(^5\)\(^-\)\(^^9\) or intramyocardial (IM) injection.\(^10\)\(^-\)\(^^12\) However, only few data exist on the efficacies of these techniques. Moreover, thus far, there is no evidence for the utility of balloon occlusion that is currently used in all clinical trials with IC cell application.

In the present study the whole-body cell distribution of autologous MNC was studied after different routes of application in pigs after MI. To allow conclusions of clinical relevance, the timing of reperfusion (4 h after infarction) and cell injection (5 days after infarction) were chosen in analogy to the settings of the recently pursued large clinical studies.\(^5\)\(^-\)\(^^9\) To test the utility of balloon occlusion, MNC retention after IC infusion without balloon (IC/noB) was compared with that after IC/B and IM injection. Initial cell kinetics were studied during IC/B and IC/noB and shortly after IM injections.

**Methods**

Protocols were approved by the regional government’s Animal Care and Use Committee (Bezirksregierung Köln, 50.203.2K 47, 15/02), and conformed with the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication No. 85-23, National Academy Press, Washington DC, revised 1996).

**Study design**

The study’s primary endpoint was cardiac retention of MNC after different routes of cell application in pigs with reperfused MI as obtained from scintigraphic whole-body scans at 1 and 24 h after cell application. Our secondary endpoints included the distribution of the transplanted cells to other organs at 1 and 24 h after application and the monitoring of the initial kinetics during the different routes of application investigated.

As there was a priori evidence for strong effects,\(^11\) a small sample size appeared to be sufficient and preferable from an ethical point of view. On the other hand, a certain minimum number of animals were needed in each group to increase the validity of measurements. The animals were assigned to the different treatment groups by lottery.

**Pigs, anaesthesia, and induction of myocardial infarction**

MI was induced in 15 male domestic pigs (age 2 months, weight 30–32 kg) by open thorax ligation of the LAD. General anaesthesia was induced by i.m. injection of azaperone 2 mg/kg and ketamine 20 mg/kg, and animals were intubated and ventilated. Anaesthesia was continued with i.v. ketamine (60 mg/kg \( \times \) h) or propofol 1% (0.5–1 mL/kg \( \times \) h). Buprenorphin (0.015 mg/kg i.v.) was given for analgesia. During anaesthesia blood oxygen saturation, exhaled CO\(_2\) and body temperature were monitored continuously. Body temperature never dropped below 37.5°C throughout the experiments. For LAD ligation the chest was opened by anterior right thoracotomy (fifth intercostal space). After longitudinal incision of the pericardium, the LAD coronary artery was visualized, and 3–5 polypropylene sutures (Ethicon, Somerville, NJ, USA) were passed twice around the vessel and tied using a tourniquet for occlusion of the distal third. The chest was surgically closed with the tourniquet being located extrathoracic for later release. Prior to LAD occlusion and 3 h afterwards an i.v. bolus of heparin (80 IU/kg) was given to avoid permanent blood clots within the infarcted artery.

**Cardiac catheterization and reperfusion**

For cardiac catheterization, the right or left femoral artery was surgically exposed and cut open with small sharp scissors. The guide wire (Emerald 0.97 mm \( \times \) 150 cm, Cordis Inc., Haan, Germany) was placed in the artery and the 6F catheterization sheath (Terumo Inc., Leuven, Belgium) was introduced and kept in place by a suture. Cardiac catheterization was performed by standard methods using a 6F Castillo 1 catheter (Cordis Inc.). The left coronary artery was depicted and the complete occlusion of the artery was confirmed. After 4 h of ischaemia the tourniquet by which the occlusion was induced was released and reperfusion was documented angiographically. Subsequently, the animals were allowed to recuperate for 5 days before cell application.

**Recovery, preparation, and labelling of mononuclear bone marrow cells with \(^{111}\)Indium**

For bone marrow aspiration, pigs were re-anæsthetized and ventilated as described above. The major trochanter was punctured and 30–50 mL bone marrow was aspirated. A Ficoll (PAA Laboratories, Pasching, Austria) gradient centrifugation was performed and the mononuclear cells were collected and washed three times with PBS. They were resuspended in 0.9% NaCl, and a mixture of 60 \( \mu \)L \(^{111}\)InCl (200 MBq) and 100 \( \mu \)L Tropolon (0.54 mg/mL) was added. After 5 min of incubation, the cells were washed twice with 0.9% NaCl. The labelling efficiency was measured with a dose calibrator (Isomed 1000, MED, Dresden, Germany) and was 64.4 ± 16.0%. Finally the cells were resuspended in PBS at a concentration of 1 × 10\(^8\) cells per 2400 \( \mu \)L for IM injections and 1 × 10\(^8\) cells per 20 mL for IC injections and stored at room temperature until use (<2 h). Cell viability was >95% as assessed by trypan blue staining immediately before cell injection. Total cellular bound radioactivity was 83.0 ± 8.5 MBq. Cell recovery and labelling and cell injection were done at the same day.

**Labelling of cells with PKH-67**

For one animal in each group, half of the cells were labelled with PKH-67 in addition to the radioactive label. The vital staining was performed by incubating the cells for 60 min in the dark at room temperature with medium containing 20 \( \mu \)M PKH-67 (Sigma-Aldrich, Steinheim, Germany), a green fluorescent cell membrane label, as described earlier.\(^114\) After that, the cells were washed twice with PBS, resuspended and mixed with the remaining half of the cells to obtain the numbers and volumes as noted above.

**Intracoronary injection of mononuclear bone marrow cells with or without balloon**

For IC injection of the cells, cardiac catheterization was performed 5 days after MI as described above using a 6F Castillo 1 guiding catheter (Cordis Inc.). An angiogram of the re-perfused infarct artery was made, a coronary guide wire (Galeo Hydro, Biotronik GmbH, Berlin,
Germany) was placed into the periphery of the LAD and an over-the-wire balloon (Concerto, 2.5 x 20 mm, Occam Int. BV, Eindhoven, The Netherlands) was placed into the coronary artery just proximal of the previous occlusion. For IC/B, the balloon was inflated with low pressure (2 atm), and one-fourth of the radiolabelled cells (i.e. 5 mL of cell suspension) were injected over 1 min. The balloon remained inflated for a total of 3 min as performed in clinical studies,4,8,15 and subsequently reperfusion was allowed for 3 min (deflated balloon). This injection procedure was repeated three more times for a total of four injections per animal. For IC/noB, the same cell injection procedure was applied, except the balloon was kept deflated during cell injection. The IC applications were done with the animals placed below the gamma camera, so the immediate kinetics of cell distribution could be observed.

Intramyocardial injection of labelled mononuclear bone marrow cell

Five days after MI pigs were re-anaesthetized and the chest was re-opened via a right intercostal incision. The infarct scar was located and exposed, and cell injection into the infarct and peri-infarct zone was performed using 1 mL syringes with 27 G needles (12 injections, 200 µL each), while the area of injection was stabilized with two top-per-holding clamps. Dynamic scintigraphic cell tracking was started few minutes after the last injection. Consecutively, the chest was surgically closed, and the 1 h whole-body scan was obtained. Then anaesthesia was stopped and the animals were allowed to recuperate from the procedure.

Scintigraphic cell tracking

Dynamic images were acquired with one image each 15 s for a total time of 30 min during IC cell injection and for 15 min shortly after IM cell injection. At 1 h, a whole-body image was obtained, which was repeated 24 h later. No later time points were investigated, since beginning from 48 h severe cell damage of 111Indium labelled cells was observed in an in vitro analysis (data not shown). A large field of view gamma camera (Picker Prism 2000, Philips Medical Systems, Best, The Netherlands) equipped with a medium-energy parallel-hole collimator was used. Energy peaks were set to 173 and 220 KeV with a 20% window and data from both windows were added to the acquisition frame. Whole-body imaging was completed after 30 min. The scintigrams were analysed in a blinded manner by a single person, who was neither involved in the randomization nor in the experimental process. At the time of sacrifice the heart was removed, cut into six transverse slices from apex to base and one-fourth of the radiolabelled cells was observed in an

Histology

For histological assessment evaluation, the hearts were harvested, cut into six short axis tissue slices and fixed in 4% paraformaldehyde in 0.1 M PBS for 24 h at 4 °C. The fixed heart slices were washed three times in PBS and tissue blocks from the regions with highest cell deposit (scintigraphy as above) were dissected and incubated in 18% sucrose solution for 6–8 h to prevent further tissue damage during the freezing process. The heart samples were frozen at −80 °C by placing them in Tissue-Tek® (Sakura Fintek, Zoeterwoude, The Netherlands). About 7 µm thick slices were prepared from frozen samples by cryo-slicing. The endothelium was stained with a selective endothelial marker Rhodamine-Griffonia (Banaderaea) Simplicifolia Lectin I (10 µg/mL in 10 mM HEPES, 0.15 M NaCl, pH 7.5, Vector Laboratories, Buringame, CA, USA) for 30 min, washed with PBS, and embedded in AquaPolyMount (Polysciences Inc., Warrington, PA, USA). Microscopy was performed using a confocal laser scanning microscope (LSM 510 META, Zeiss, Gottingen, Germany).

Data analysis and statistical analysis

The data obtained from dynamic scintigraphies during IC cell injections were fitted into one-phase exponential decay curves, and cell loss after IM cell injection was fitted to a linear curve using Prism® (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were done with InStat® (GraphPad Software Inc.) applying AUC comparisons (Figure 3), one-way analysis of variance (ANOVA) with the Tukey–Kramer multiple testing correction to account for the inflation of the Type I error (Figure 4). In addition, the P-values from the multiple F-tests in Table 2 were adjusted according to the Bonferroni method. The usual significance level of 0.05 was used and all tests were two-sided. All variables are summarized by mean ± SD.

Results

Of the 15 animals used for this study, 1 animal died after infarction, 1 animal died after IM injection, and 1 animal had insufficient reperfusion in control angiography. These three animals were excluded from analysis. The remaining 12 followed the protocol until the end: 4 animals received IC/noB, 4 animals received IC/B, and 4 animals received IM cell injections. Invasive blood pressures were measured during cardiac catheterization at the time of reperfusion and at the time of IC cell injection and no differences between groups were observed. However, blood pressure (systolic, diastolic, and mean) was higher at the time of cell injection than at the time of reperfusion (systolic 97 ± 8 vs. 74 ± 3 mmHg, P = 0.008; diastolic 67 ± 7 vs. 49 ± 3 mmHg, P = 0.02; mean 82 ± 7 vs. 60 ± 3 mmHg, P = 0.006).

Kinetics of cell deposit and loss after intracoronary cell injection without balloon vs. with balloon and intramyocardial injection

The kinetic analysis revealed two phases of cell decay after each of the four IC cell injections with balloon: phase 1 immediately after cell injection with the balloon inflated (no-flow) and phase 2 after balloon deflation (re-flow), which appeared to turn into a slow cell decay after termination of the injection cycles (Figures 1A and 2). Each of the four IC cell injections with balloon resulted in a peak of radioactivity in the heart immediately after injection, followed by the phase 1 cell decay, a one-phase exponential decay (even though no coronary flow was allowed by the inflated balloon) with a mean half-time of 18.9 ± 3.3 min (4 animals, 4 injections each). There was no change in half-times and the corresponding time constants tau with subsequent injections. Upon balloon deflation (re-flow), a second one-phase exponential cell loss was observed (phase 2, mean half-time 28.0 ± 15.6 s), again without change in half-times and tau with subsequent injections (Table 1). It has to be noted that half-times and time constants tau were dependent on the maximal cell loss observed in each...
phase, therefore they do not reflect the faster cell loss during reperfusion.

Immediately after IC/noB inflation, a one-phase exponential decay of cell number within the heart was observed with a mean half-time of $73.4 \pm 70.4$ s (4 animals, 4 injections each) (Figures 1B and 2). Similar to the IC/B injections no changes in half-times and time constants $\tau$ were observed with subsequent injections after IC/noB (Table 1).

In contrast to the IC injections (both IC/noB and IC/B), after IM cell injection no exponential phase of cell loss was observed (Figure 1C and 2). Instead, only a single slow and linear cell decay was seen (following the equation $y = -0.000027 \times x + 0.339532$, $R^2 = 0.96$). It has to be noted that in the IM group, in contrast to IC, scintigraphy could only be started few minutes after cell injection, so that a short early phase of exponential cell loss may have been missed.

**Figure 1** Representative dynamic scintographies of hearts (P-A projection) after injections of radiolabelled autologous mononuclear bone marrow cells into infarcted swine hearts. (A) Intracoronary injection with balloon during balloon inflation (1–3 min) and after balloon deflation (4–6 min). (B) Intracoronary injection without balloon and (C) intramyocardial cell injection.

**Cell deposition and loss after intracoronary cell injection without balloon vs. with balloon**

For both IC/B (Figure 3A) and IC/noB (Figure 3B), peak cell deposit and cell retention at 6 min increased with each subsequent IC cell injection (all $P$ for trend $<0.0001$). While overall peak cell deposit was higher after IC/B than after IC/noB cell injection ($P < 0.05$), cell retention at 6 min did not differ between groups ($P = 0.2$, Figure 3A and B).

**Cell distribution 1 and 24 h after cell injection**

One hour after cell injection whole-body scan revealed that 4.1 ± 1.1% of total radioactivity was located in the heart after IC/noB application vs. 6.1 ± 2.5% after IC/B ($P = 0.19$) and 20.7 ± 2.3%
Figure 2. Scintigraphic analysis of cell persistence of the first 30 min during and after intracoronary bone marrow cell injection (intracoronary injection with and without balloon) and the first 15 min after intramyocardial injection. After each intracoronary cell injection with balloon (closed diamond) two one-phase exponential cell decays were observed [phase 1 with the balloon inflated (no-flow), phase 2 with the balloon deflated (re-flow)]. After each intracoronary cell injection without balloon (open diamond) one one-phase exponential cell decay was seen with a lower peak cell deposit but with the same cell retention at the end of each cycle as after intracoronary injection with balloon. After intramyocardial cell injection (closed circle) only one slow linear phase of cell decay was observed (registration started few minutes after end of injection). Data are shown as mean values.

Table 1. Half-times (time constants tau $\tau$) for one-phase exponential decays in seconds after intracoronary injection with balloon vs. intracoronary injection without balloon

<table>
<thead>
<tr>
<th>Phase 1 (no-flow)</th>
<th>Phase 2 (re-flow)</th>
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<tbody>
<tr>
<td><strong>IC/B ($n = 4$)</strong></td>
<td><strong>IC/noB ($n = 4$)</strong></td>
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<tr>
<td>Injection 1</td>
<td>18.7 ± 4.5 [(\tau = 27.0 \pm 6.5)]</td>
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<tr>
<td>Injection 2</td>
<td>17.8 ± 10.2 [(\tau = 25.7 \pm 14.7)]</td>
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<tr>
<td>Injection 3</td>
<td>22.5 ± 6.5 [(\tau = 32.5 \pm 12.3)]</td>
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<tr>
<td>Injection 4</td>
<td>16.4 ± 2.3 [(\tau = 23.6 \pm 3.4)]</td>
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<tr>
<td>Mean of Injections 1–4</td>
<td>18.9 ± 6.8 [(\tau = 27.2 \pm 9.8)]</td>
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</table>

after IM injection ($P < 0.001$ vs. IC/noB and IC/B). After 24 h the numbers were 3.0 ± 0.6% after IC/noB injection, 3.3 ± 0.5% after IC/B injection ($P = 0.43$), and 15.0 ± 3.1% after IM ($P < 0.001$ vs. IC/noB and IC/B) (Figure 4). Regarding the distribution of grafted cells to extracardiac organs, overall the same pattern of distribution at 1 and 24 h was observed for all application
Figure 3 Comparison of cell deposit after intracoronary infusion with balloon (A) and intracoronary infusion without balloon (B) of radio-labelled mononuclear bone marrow cells. The black bars represent each injection’s initial maximum (approximately at 1 min after start of injection), the medium grey bars represent the cell retention immediately before balloon deflation (end of phase 1, at 3 min after start of injection), and the light grey bars represent the cell retention at 6 min after start of injection (end of phase 2). Data are shown as mean ± SD.

Figure 4 Quantitative analysis of cardiac cell persistence at 1 h and 24 h after intracoronary cell injection with balloon (closed diamond), intracoronary cell injection without balloon (open diamond), intramyocardial injection (closed circle) as a fraction of cardiac radioactivity of total body radioactivity. Data are shown as mean ± SD.
Table 2  Organ distribution of 111Indium-labelled mononuclear bone marrow cells after injection into infarcted/reperfused pig hearts (intracoronary injection with balloon vs. intracoronary injection without balloon vs. intramyocardial injection)

<table>
<thead>
<tr>
<th></th>
<th>IC/B (n = 4)</th>
<th>IC/noB (n = 4)</th>
<th>IM (n = 4)</th>
<th>P-value</th>
<th>P-value</th>
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<tr>
<td></td>
<td>1 h</td>
<td>24 h</td>
<td>1 h</td>
<td>24 h</td>
<td>1 h</td>
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<tr>
<td>Total, %</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Heart, %</td>
<td>6.1 ± 2.5</td>
<td>3.3 ± 0.5</td>
<td>4.1 ± 1.1</td>
<td>3.0 ± 0.6</td>
<td>20.7 ± 2.3</td>
</tr>
<tr>
<td>Lung, %</td>
<td>47.9 ± 7.0</td>
<td>50.1 ± 9.2</td>
<td>42.4 ± 5.3</td>
<td>39.6 ± 5.2</td>
<td>42.0 ± 4.7</td>
</tr>
<tr>
<td>Liver, %</td>
<td>22.5 ± 8.6</td>
<td>24.0 ± 4.5</td>
<td>25.9 ± 3.7</td>
<td>26.8 ± 2.8</td>
<td>14.6 ± 5.3</td>
</tr>
<tr>
<td>Spleen, %</td>
<td>4.5 ± 2.3</td>
<td>5.6 ± 2.5</td>
<td>6.7 ± 4.0</td>
<td>6.4 ± 3.6</td>
<td>4.2 ± 2.5</td>
</tr>
<tr>
<td>Thorax, %</td>
<td>54.1 ± 9.5</td>
<td>53.4 ± 9.4</td>
<td>46.5 ± 5.8</td>
<td>42.5 ± 5.7</td>
<td>62.7 ± 5.4</td>
</tr>
<tr>
<td>Abdomen, %</td>
<td>30.9 ± 8.6</td>
<td>34.2 ± 6.3</td>
<td>37.4 ± 3.9</td>
<td>38.2 ± 4.2</td>
<td>23.7 ± 7.8</td>
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Scintigraphy of heart slices and histological analysis of grafted cells

Scintigraphy of the heart slices revealed that for both IC/noB and IC/B, most of the injected MNC remained in that region which is perfused by the distal LAD, equalling the infarcted region (apex, septum) (Figure 6A–C). After IM injection a similar observation was made, i.e. the cells remained mainly in the cardiac region in which they were injected (mostly the apex), but at much higher concentration, leading to a very intense signal of the apex which they were injected (mostly the apex), but at much higher concentration, leading to a very intense signal of the apex (Figure 6D). Confocal microscopy in cryosections from those regions with highest grafter cell concentration revealed very distinct patterns of cell localization depending on the mode of injection: After IC injection almost all of the PKH-67 labelled cells were found within the vascular structures as depicted by lectin I stain (Figure 7A and B) without differences between IC/noB and IC/B. Only very rarely an injected cell was found in the interstitium of the heart. In contrast, the opposite observation was made for PKH-67 labelled cells after IM injection: All of the cells were located in the interstitium of the heart (Figure 7C and D), whereas no cells were detected co-localized with the vasculature.

Discussion

In all larger clinical studies to date, IC cell injection is performed with balloon occlusion of the artery proximal to the injection site to achieve longer contact time of the injected cells and to facilitate homing of the cells.5–9 This procedure is chosen based on mere assumption of its efficacy, but on the other hand it is even conceivable that the hyperemia following each cycle of balloon-induced ischaemia may have an adverse effect on cell deposit. The present study is the first to systematically compare cell retention after IC/B and IC/noB injection. Most interestingly, only the peak of cell deposit early after each injection was higher in the IC/B group than in the IC/noB group, but no difference was observed neither at the end of injection cycles nor at 1 and 24 h after cell injection. If short or longer term cell persistence (and not short-time peak deposit during injection together with ischaemia reperfusion post-conditioning by balloon inflation) is important for IC MNC therapy to be successful, our data suggest, that IC injection of MNC can be done equally effective without balloon inflation. However, this hypothesis needs further verification. Specifically, the mode by which MNC injection improves cardiac function needs to be clarified to understand whether mid-term cell retention or short-term peak cell deposit are important.

Another interesting finding of this study is that fractionated IC cell injection, as used in all larger clinical studies to date, is indeed efficient for cell deposition and leads to an accumulation of cells within the heart. However, it remains unknown whether this repetitive procedure is more efficient than one single large bolus injection of the cells. Also in the interest of further simplifying the methodology of cell therapy, it will be interesting to explore this question in future experiments.

Overall, grafting efficacy was rather low after IC injection of cells with balloon with 6% persistent cells after 1 h and 3% after 24 h (IC/noB: 4% at 1 h, 3% at 24 h). Similar grafting efficacies were found in the clinical BOOST study (2.9 ± 0.6% after 1 h, no longer term data available)16 and after 2 h (5%) and 18 h (1%) in a clinical case report,17 in which also MNC were used and the times until reperfusion and times of cell injection after MI were similar as in our experimental study. Interestingly, the numbers observed here at 1 h after IC injection are, albeit within the same order of magnitude, somewhat higher than the numbers observed by Hou et al. who found 2.6 ± 0.3% cells retained after IC injection in infarcted pig hearts.13 However, different cell types (autologous bone marrow MNC in our study vs. peripheral blood MNC from humans), times of coronary occlusion to induce MI (4 h in the present study vs. 45 min) and different numbers of IC injections (4 injections here vs. one single injection) were used. Which of the different conditions is responsible for the higher yield in grafted cells remains unclear. We can only speculate that the longer time of ischaemia or the higher percentage of immature cells in the bone marrow mononuclear fraction (vs. the peripheral blood mononuclear fraction) may contribute. Hofmann et al. noted a higher grafting efficacy when they used a cell population enriched for CD34 positive cells,16 which

Table 2  Organ distribution of 111Indium-labelled mononuclear bone marrow cells after injection into infarcted/reperfused pig hearts (intracoronary injection with balloon vs. intracoronary injection without balloon vs. intramyocardial injection)
may indicate that the higher percentage of immature cells in bone marrow MNC vs. peripheral blood MNC (e.g. CD34: 0.1–1 vs. 0.01%) may play a role for higher cell engraftment. A relatively high number of persisting cells was also found for mesenchymal stem cells (6%) 14 days after IC injection in a recent study in pigs with MI. One reason for that finding might be the plugging of grafted mesenchymal bone marrow cells in coronary arterioles with reduction in coronary artery flow upon cell injection which was seen in that study, and which was also described earlier in healthy dogs. On the other hand, the potential of MSC to migrate across the endothelial barrier might also contribute to higher cell persistence.

In accord with previous observations, we found that IM injection of MNC is more effective than IC injection at 1 h. This difference is sustained for at least 24 h. With the timing of reperfusion (4 h occlusion), cell harvesting and cell injection (5 days after infarction) being adapted from the typical protocols from recent clinical trials on cell therapy for acute MI, we assume that the cell persistence after IC and IM injection are similar in the clinical setting. Hou et al. were the first to compare the grafting efficacy between IC injection of mononuclear cells (albeit from peripheral blood) and IM injection in pigs. They found that grafting is more effective after IM injection than after IC injection (11.3 ± 3 vs. 2.6 ± 0.3% at 1 h). Although our numbers were higher (22% at 1 h after IM injection, Figure 5 Quantitative analysis of organ distribution of grafted mononuclear bone marrow cells 1 h and 24 h after intracardiac cell injection (A) intracoronary infusion with balloon, (B) intracoronary infusion without balloon, (C) intramyocardial injection. Data are shown as mean ± SD.)
Interestingly, no difference in cell persistence between the IM and IC injection was observed, which was much slower and could be fit to a linear decay. However, due to experimental constraints the IM injection could not be done under the gamma camera, and the first minutes of cell kinetics could not be monitored for this condition. Most likely, also after IM injection of the cells, the cell decay follows an exponential curve, and probably many cells are lost due to immediate leakage from the injection sites as shown previously in small animal studies. To further study the early kinetics of IM injection in large animals scintigraphically, other techniques like catheter-based endomyocardial cell injection may be superior to the open-chest technique used in the present study.

Interestingly, the slow parts of cell loss after IC/B and IC/noB injection (after 24 min) and after IM injection seem to have similar slopes, indicating that at some point the kinetics of cell loss become independent from the mode of cell application. Our histological analysis of cell localization clearly demonstrates that this cannot be explained by residence of cells in similar compartments within the cardiac tissue. In fact, very distinct localizations of MNC were found dependent on the mode of cell injection with the cells residing mostly in the vasculature without crossing the endothelial barrier after IC injection and with the cells residing exclusively in the interstitium outside the vessels after IM injection. The first observation nicely matches previous investigations in which human MNC and circulating endothelial progenitor cells were found to interact with the endothelium, adhere and putatively integrate, but were in no case found to migrate across the endothelial barrier of rat aorta or the coronary arteries of Langendorff-perfused mouse hearts.

The results on organ distribution of injected cells show some differences to previous observations in man and rats: In humans, the majority of cells outside the heart were localized in liver and spleen at 1 h (>85%), and persisted until 18 h. In rats, even some evidence for MNC proliferation in the spleen was found. In contrast, here, only a small fraction of cells was found in the spleen at 1 and 24 h, whereas about half of the cells were localized in the lungs both immediately after injection and at 24 h—independent from mode of application. The circulatory and perfusion perspective offers an easy explanation for this significant cell trapping in the lungs, which are perfused by 100% of cardiac output. Interestingly, the 24 h organ distribution after IC injection very closely reflects the fraction of cardiac output that these organs are being perfused with. Nevertheless, the differences in organ distribution between our study and those in humans are striking. Since other observations in pigs, albeit with different cell types, showed similar results as ours, it seems likely that significant species differences between humans and pigs are relevant for organ distribution of MNC, which may involve the properties of mononuclear cells recovered from the bone marrow or the capillary properties of the lungs, the spleen, and/or the liver.

To date, the role of persistence and organ distribution of grafted bone marrow cells for the therapeutic benefit of cell therapy after...
MI is unclear. In contrast to cell replacement approaches with isolated contractile cardiac or skeletal myocytes, it may well be that only few persisting bone marrow (stem) cells are needed for therapeutic success, and only a short contact time of cells with the injured myocardium may suffice. It is also still unclear whether cells that are deposited interstitially as done by IM injection have the same therapeutic potential as cells that are deposited via the small capillaries of the vascular tree as done by IC injection. In addition, it may be plausible that the body distribution of cells with a temporarily increased number of circulating immature cells and residence of grafted cells in organs other than the heart may have a systemic impact on the composition and concentrations of serum cytokines which in turn may enhance regeneration or recovery of the injured heart. However, to further elucidate the mode of action of bone marrow cell transfer, understanding the fate of grafted cells is one important step. Our data together with previous studies suggest that, if cell persistence is needed for improvement of cardiac function and distribution to other organs is not useful or even harmful, IM injection of cells is superior to IC injection.

As limitation it needs to be noted that, albeit unlikely, it cannot be excluded that the ‘unphysiological’ mode of infarct induction by open-chest coronary ligation may have an influence on the observed results. Furthermore, in the current study we have monitored distribution of radioactivity as a surrogate for cell distribution, however this might be associated with some inaccuracies, e.g. due to leakage of radioactive label from the cells.

In summary, in the present study cell distribution and cell persistence in the heart was quantified after mononuclear cell injection into infarcted pig hearts under conditions which are most relevant to clinical treatment algorithms. The conditions used in the present animal study (duration of ischaemia, cell type, timing of cell injection) were chosen to be as similar as possible to those applied in most clinical studies of IC injection for acute MI. For the first time, the immediate kinetics of cell wash-out were visualized during IC cell application with or without balloon. After IC cell injection with balloon, we found that two phases of exponential cell decay could be distinguished (during inflated balloon and after deflation), whereas it was only one phase after IC injection without balloon. Early cell deposit was more effective with balloon than without balloon, but as soon as 6 min after cell injection this difference was no longer present. The cell loss after IC injection was much faster and more pronounced than after IM injection. In consequence, cell deposit was more effective after IM injection of cells compared with IC, and our data show that this difference is not transient but sustained for at least 24 h. The majority of cells is washed out and carried away to other organs with the lung hosting ~50% of the grafted cells, the liver 10% and the spleen 5%, independent from mode of application. In conclusion, if longer term cell persistence after IC cell delivery, injections without balloon are equally effective as those with balloon occlusion. Cell distribution to other

Figure 7  Confocal laser scanning microscopy of the cardiac regions with highest deposit of grafted mononuclear bone marrow cell as estimated by scintigraphy after intracoronary injection of the cells (A and B), and after intramyocardial injection of the cells (C and D). Green, mononuclear bone marrow cell; red, endothelium stained with lectin I.
organisms is relevant in both application techniques and must be considered in all approaches of cardiac cell therapy.

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**References**