Clinical research

Cellular repopulation of myocardial infarction in patients with sex-mismatched heart transplantation

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Aims Recent studies have suggested that human extracardiac progenitor cells are capable of differentiating into cardiomyocytes. In animal studies, myocardial infarction attracted bone marrow stem cells and enhanced their differentiation into cardiomyocytes. Based on these findings, we hypothesised that myocardial infarction stimulates the invasion of progenitor cells and their differentiation into endothelial and cardiac cells in the human heart.

Methods and results We compared autopsy samples from male control patients who had received a female donor heart with samples from such patients who developed myocardial infarction after transplantation. Fluorescence in situ hybridisation (FISH) for detection of the Y-chromosome was combined with immunofluorescence staining for CD45 and CD68 to distinguish host-derived inflammatory cells. Additionally, we used a 3D-confocal imaging technique to indisputably assign Y-chromosome-positive nuclei to their cytoplasm. In patients with myocardial infarction after heart transplantation (n = 5), host-derived non-inflammatory progenitor and endothelial cells were significantly increased compared to non-infarcted patients (n = 9). Yet, by using this novel multi-step approach, only 0.02% of all cells were estimated to be male cardiomyocytes and their increase in infarcted regions to 0.07% was not significant.

Conclusion Myocardial infarction enhances the invasion of extracardiac progenitor cells and their regeneration of endothelial cells. However, a significant differentiation into cardiomyocytes as a physiological mechanism of postischaemic regeneration does not occur in transplanted patients.

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KEYWORDS
Stem cells;
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Cardiac regeneration;
Three-dimensional confocal microscopy

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Regeneration of cardiomyocytes has been assumed not to occur in the human heart. Recent studies in patients after heart or bone marrow transplantation, however, have indicated that cardiomyocyte repopulation by extracardiac progenitors takes place.\(^1\) Yet, there is great controversy regarding the frequency of cardiomyocyte differentiation from invaded stem cells. In addition, it is still unclear which subpopulations of progenitor cells are capable of differentiation into cardiomyocytes. Further characterisation of these cells may allow the identification of a subpopulation of extracardiac progenitor cells that is capable of regenerating myocardium in vivo under physiological circumstances.

Potential candidates could be mesenchymal and/or haematopoietic stem cells or a special subgroup of these cells. Both have been shown to have great plasticity, with the ability to differentiate in vitro into many different lineages, such as skeletal muscle, neuronal cells, chondrocytes, adipocytes, and osteocytes.\(^5\) When injected into mouse myocardium, these cells differentiate into cardiomyocytes.\(^6\)\(^7\)

In addition, identification of factors regulating the migration and differentiation of stem cells is of great importance. In the mouse infarction model, Orlic et al.\(^7\) showed that haematopoietic stem cells were attracted to the infarcted region, and that they rapidly differentiated into myocardium, resulting in significant recovery of muscle mass after infarction.

Based on these findings, we asked whether acute myocardial infarction enhances invasion of injured myocardium with stem cells, possibly from the bone marrow, and whether differentiation of stem cells into cardiomyocytes is increased in the infarcted human heart. To address this question we investigated autopsy samples from male patients who had undergone cardiac transplantation with a female donor heart and suffered from myocardial infarction after transplantation. In order to analyse the origin and differentiation of cells, Y-chromosomes were detected by FISH, inflammatory and endothelial cells by immunohistochemistry, and myocyte differentiation by morphology using fluorescence and confocal microscopy. Autopsy samples from patients with and without myocardial infarction were compared.

**Methods**

**Patients**

In a review of the transplantation files of the Heart Centre Bad Oeynhausen, Germany, we identified 432 male patients who had received female donor hearts. Out of the 432 patients, 35 had clear documentation of myocardial infarction. We excluded all questionable cases. We chose to use autopsy samples rather than small biopsies since only the use of larger blocks of tissue would allow us to carefully identify regions of infarction, rejection, and normality, and to check a larger area for the presence of Y-chromosomes. Autopsy samples were available from 20 of these patients, and we also examined autopsy material from 15 patients who had no myocardial infarction after sex-mismatched heart transplantation. Each of the 35 samples was classified according to ISHT criteria.\(^8\) Six patients showing strong levels of rejection were excluded from analysis. In 13 patients, a successful combination of FISH and immunofluorescence could not be performed according to our quality standards. Of the 16 remaining patients, 5 had developed myocardial infarction after heart transplantation more than 24 h prior to death. The age of infarction was estimated according to histopathological criteria (Table 1). Nine patients without myocardial infarction after heart transplantation and with a clearly documented cause of death were analysed in this study (Table 2).

Similarly processed heart tissue from five different non-transplanted male and three female patients obtained from the department of pathology of Göttingen served as negative and positive controls, respectively. One sample of each patient was analysed. These samples were used to check the specificity and efficiency of FISH. The study design was submitted to the internal review board of the University of Göttingen; no objections were raised.

**Preparation of tissue samples**

All heart specimens were fixed in 4% PBS-buffered formalin and were paraffin-embedded according to standard procedures. Two-micron sections were prepared and placed on Teflon-coated slides.

**Fluorescence in situ hybridisation**

Common FISH protocols require the use of proteinases, which impair the simultaneous detection of cell surface markers by immunophenotyping. Therefore, the FISH protocol as provided by Vysis, USA was modified: after deparaffinisation, sections were treated with 2 × SSC for 10 min at 75 °C. Instead of enzymatic digestion by proteinase K, slides were then pre-treated by microwave boiling in citrate buffer (Antigen Retrieval Citra, BioGenex, San Ramon, USA) for 10 min. Slides were rinsed with water and 2 min with 2 × SSC, and then were air-dried in the dark. They were incubated with the probe (CEP X SG/CEP Y (alpha) SO DNA Probe, Vysis, USA) for 5 min at 75 °C and then at 37 °C in a humid chamber overnight. Slides were washed in 2 × SSC at 73 °C for 2 min. Nuclei were counterstained with DAPI II (Vysis, USA) or SYBR Green (Molecular Probes, USA).

**Immunofluorescence**

After successful hybridisation with the X and Y-chromosome was confirmed, slides were washed twice with PBS, blocked with 1% BSA in PBS, and incubated with a mix of mouse-anti-human leukocyte-common-antigen CD45 (1:50, Dako, Denmark) and mouse-anti-human macrophage-antigen CD68 (1:50, Dako, Denmark) or with antibodies for von Willebrand Factor (1:50, FITC-conjugated AffiniPure Goat-anti-mouse IgG H + L, Jackson Immuno Research Laboratories) for another hour at 37 °C. Coverslips were applied to slides with Vectashield Mounting Medium (Vector, Burlingame, CA, USA).

We chose to use CD45 and CD68 to discriminate inflammatory from non-inflammatory CD45+/CD68− progenitor cells. CD45 is one of the most abundant leukocyte cell-surface glycoproteins and is expressed exclusively on cells of the haematopoietic system.\(^9\) Expression of CD68 (macrolailin) is a characteristic feature of macrophages.\(^10\) Because many macrophages are attracted to infarcted tissue, we used a mix of both markers to identify the actual percentage of non-inflammatory progenitor...
Morphometric analysis of the hybridised and immunostained groups: (1) CD45 and expression of CD45 and/or CD68 into one of the 8 following mean of 765. All cells were classified according to morphology of nuclei counted per slide ranged from 285 to 1022, with a

nm

þ

(4) endothelial cells. Each of these groups was further classified

endothelial cell, non-cardiomyocyte), (3) cardiomyocytes, and

nm; 485/17 nm; 560/18 nm, beam splitter TFT 440 nm

Spectrum Orange at the same time, the Triple filter (Ex 360/51

nm, beam splitter 565 nm LP and Em 590nm LP; and for DAPI

Em 535/40 nm BP; for Spectrum orange fluorophore Ex 535/50

Green fluorophore Ex 480/30 nm, beam splitter 505 nm LP and

Olympus). We used the following filter sets: for FITC/Spectrum

Tissue analysis

Morphometric analysis of the hybridised and immunostained sections was performed with an Olympus BX51 fluorescence microscope equipped with a 100-W mercury vapour lamp at a magnification of 100× (objective UPlanAPO 100×/1.35 oil iris, Olympus). We used the following filter sets: for FITC/Spectrum Green fluorophore Ex 480/30 nm, beam splitter 505 nm LP and Em 535/40 nm BP; for Spectrum orange fluorophore Ex 535/50 nm, beam splitter 565 nm LP and Em 590nm LP; and for DAPI fluorophore Ex 358/55nm, beam splitter 395 nm and Em 420 nm LP. In addition, for the detection of DAPI/ SpectrumGreen/ Spectrum Orange at the same time, the Triple filter (Ex 360/51 nm; 485/17 nm; 560/18 nm, beam splitter TFT 440 nm + 500 nm + 570 nm, Em TBP 460 nm + 520 nm + 600 nm) was used.

Twenty visual fields were analysed on each slide. The number of nuclei counted per slide ranged from 285 to 1022, with a mean of 765. All cells were classified according to morphology and expression of CD45 and/or CD68 into one of the 8 following groups: (1) CD45 and/or CD68, (2) CD45 and CD68 (non-endothelial cell, non-cardiomyocyte), (3) cardiomyocytes, and (4) endothelial cells. Each of these groups was further classified as either Y-chromosome-positive or Y-chromosome-negative.

The evaluation was made according to the following criteria: cells with a clear green margin or green cytoplasm were classified as CD45 and/or CD68 (i.e., inflammatory cells). Cells located along the inside of an arteriole with little cytoplasm and an elongated nucleus were classified as endothelial cells. In additional stainings, endothelial cells were confirmed by immunofluorescence using antibodies against vWF.

To be classified as a cardiomyocyte, cells had to fulfil the common histological criteria: cells were notably larger in size with a centrally located nucleus and typical striated cytoplasmic structure. To verify our criteria for cardiomyocytes, we also performed immunofluorescence with antibodies against the cardiac-specific proteins titin and calsequestrin in control samples of human myocardium.

To determine whether a nucleus belonged to a cardiomyocyte, strict criteria were applied: the nucleus had to be fully surrounded by, and in the same focal plane, as the cytoplasm of the cardiomyocyte, and it had to be negative for both CD45 and CD68. Staining for the Y-chromosome was regarded as positive if a punctate orange signal was present within a given blue-stained nucleus and in the same focal plane. Additionally, single band pass filters for FITC and Spectrum orange were used to ensure that the orange signal of the Y-chromosome was not caused by the autofluorescence of lipofuscin. (Auto fluorescence could be seen in both filters, the Y-chromosome only in the Spectrum orange filter.)

Areas containing artefacts were excluded, for instance, those caused by the cutting procedure, as well as overlapping nuclei. Furthermore, cells located within vessels were not counted.

cells, negative for both CD45 and CD68. Detection of a Y-chromosome within a nucleus of a von Willebrand factor (vWF) positive cytoplasm/cell membrane was considered a host-derived endothelial cell.

Table 1 Patients with myocardial infarction

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Survival after HTX</th>
<th>Age of infarction(s)</th>
<th>Region of infarction</th>
<th>Rejection (ISHLT)</th>
<th>Cause of death</th>
<th>Y+ of total, %/n</th>
<th>Y+, CD45+, CD68+, %/n</th>
<th>Y+, end. cells, %/n</th>
<th>Y+, CM, %/n</th>
<th>Cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58 y</td>
<td>38 m</td>
<td>1 d and 2 w</td>
<td>Posterior wall</td>
<td>0</td>
<td>Peritonitis, septic shock</td>
<td>39.2/112</td>
<td>13.3/38</td>
<td>0.4/1</td>
<td>0.4/1</td>
<td>285</td>
</tr>
<tr>
<td>2</td>
<td>53 y</td>
<td>26 m</td>
<td>3–4 w</td>
<td>Posterior wall, papillary muscle</td>
<td>1B</td>
<td>Cardiogenic shock</td>
<td>38.2/241</td>
<td>9.2/58</td>
<td>0.5/3</td>
<td>0.5/3</td>
<td>631</td>
</tr>
<tr>
<td>3</td>
<td>62 y</td>
<td>1 m</td>
<td>3–4 w</td>
<td>Anterior wall</td>
<td>0</td>
<td>Cardiogenic shock</td>
<td>10.4/46</td>
<td>2.0/9</td>
<td>1.6/7</td>
<td>0.0</td>
<td>442</td>
</tr>
<tr>
<td>4</td>
<td>70 y</td>
<td>6 y</td>
<td>3–4 w</td>
<td>Septum</td>
<td>1A</td>
<td>Cardiogenic shock</td>
<td>8.9/61</td>
<td>2.1/14</td>
<td>0.3/2</td>
<td>0.0</td>
<td>683</td>
</tr>
<tr>
<td>5</td>
<td>62 y</td>
<td>5 w</td>
<td>1 d and 3–4 w</td>
<td>Posterior wall</td>
<td>0</td>
<td>Cardiogenic shock</td>
<td>23.8/206</td>
<td>2.8/24</td>
<td>0.1/1</td>
<td>0.2/2</td>
<td>864</td>
</tr>
</tbody>
</table>

y, year; m, month; w, week; d, day; HTX, heart transplantation; end. cells, endothelial cells; CM, cardiomyocytes.

Within one region, two different ages of infarction were identified.

Table 2 Patients without myocardial infarction

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Survival after HTX</th>
<th>Analysed region</th>
<th>Rejection (ISHLT)</th>
<th>Cause of death</th>
<th>Y+ of total, %/n</th>
<th>Y+, CD45+, CD68+, %/n</th>
<th>Y+, end. cells, %/n</th>
<th>Y+, CM, %/n</th>
<th>Cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65 y</td>
<td>12 d</td>
<td>Septum</td>
<td>0</td>
<td>Liver failure</td>
<td>2.6/20</td>
<td>1.2/9</td>
<td>0.0</td>
<td>0.0</td>
<td>776</td>
</tr>
<tr>
<td>2</td>
<td>67 y</td>
<td>3 m</td>
<td>Anterior wall</td>
<td>0</td>
<td>Haemorrhagic shock</td>
<td>9.3/89</td>
<td>1.9/18</td>
<td>0.1/1</td>
<td>0.0</td>
<td>958</td>
</tr>
<tr>
<td>3</td>
<td>62 y</td>
<td>5 h</td>
<td>Septum</td>
<td>0</td>
<td>Cardiogenic shock</td>
<td>4.3/41</td>
<td>1.7/16</td>
<td>0.0</td>
<td>0.1/1</td>
<td>964</td>
</tr>
<tr>
<td>4</td>
<td>61 y</td>
<td>5 d</td>
<td>Anterior wall</td>
<td>0</td>
<td>Cardiogenic shock</td>
<td>2.5/19</td>
<td>1.7/13</td>
<td>0.0</td>
<td>0.0</td>
<td>753</td>
</tr>
<tr>
<td>5</td>
<td>72 y</td>
<td>20 d</td>
<td>Posterior wall</td>
<td>0</td>
<td>Multi organ failure</td>
<td>5.2/42</td>
<td>2.4/19</td>
<td>0.0</td>
<td>0.0</td>
<td>809</td>
</tr>
<tr>
<td>6</td>
<td>66 y</td>
<td>3 y, 10 m</td>
<td>Septum</td>
<td>0</td>
<td>Haemorrhagic shock</td>
<td>4.1/42</td>
<td>1.4/14</td>
<td>0.0</td>
<td>0.0</td>
<td>1022</td>
</tr>
<tr>
<td>7</td>
<td>59 y</td>
<td>8 y, 9 m</td>
<td>Anterior wall</td>
<td>0</td>
<td>Multi organ failure</td>
<td>12.1/91</td>
<td>2.0/15</td>
<td>0.0</td>
<td>0.3/2</td>
<td>754</td>
</tr>
<tr>
<td>8</td>
<td>72 y</td>
<td>4 y, 4 m</td>
<td>Posterior wall</td>
<td>7/A</td>
<td>Cardiogenic shock</td>
<td>3.7/36</td>
<td>1.1/11</td>
<td>0.0</td>
<td>0.0</td>
<td>984</td>
</tr>
<tr>
<td>9</td>
<td>60 y</td>
<td>7 y, 2 m</td>
<td>Anterior wall</td>
<td>0</td>
<td>Haemorrhagic shock</td>
<td>4.3/34</td>
<td>1.3/14</td>
<td>0.0</td>
<td>0.0</td>
<td>785</td>
</tr>
</tbody>
</table>

y, year; m, month; d, day; HTX, heart transplantation; end. cells = endothelial cells; CM = cardiomyocytes.

Rejection was circumscribed, within the analysed region, no lymphocyte infiltrate was present.
In samples from patients with myocardial infarction, cell evaluation was performed within infarcted areas that were adjacent to intact tissue. This was defined by the presence of ischaemic lesions and adjacent intact tissue in the same visual field (at 20× magnification). Only tissue within a range of 300 μm of infarcted tissue was analysed. An example of infarcted tissue is shown in Fig. 1(a). In tissue samples from patients without myocardial infarction, only areas with morphologically fully intact myocardium were analysed (Fig. 1(b)).

Ten cells that fulfilled the criteria for Y-chromosome-positive cardiomyocytes by fluorescence microscopy (see above) were additionally analysed by confocal microscopy (Leica TCS confocal laser scanning system with microscope DM IRB) for three-dimensional analysis by computer-supported rotation of the cells.

Statistical analysis

Data are given as mean ± SEM. Statistical differences were calculated with the Student t test for unpaired samples. \( P < 0.05 \) was considered to be statistically significant.

Results

Sensitivity and specificity of FISH and immunostaining in transplanted human hearts

We first aimed to establish a methodology that combined detection of Y-chromosomes in cardiomyocytes at a high sensitivity and sufficient specificity to minimise artefacts due to overlapping of cells. Previous studies, which used a similar approach, reported to detect Y-chromosomes in 34–55% of male cells in sections ranging from 4–6 μm. Here we used sections that were substantially thinner (2 μm) to increase specificity when using conventional microscopy. In normal hearts of non-transplanted male patients (positive controls), 57% of cardiomyocyte nuclei and 65% of nuclei of other cells contained a detectable Y-chromosome. In normal hearts from non-transplanted female patients (negative controls), no fluorescence signal indicating a Y-chromosome was detected. Thus, the FISH method utilised in this study combined superior sensitivity with enhanced specificity (due to thinner sections), as compared to methods used in previous studies. Since the sensitivity for detection of Y-chromosomes using this assay is less than 100%, the real numbers of Y-chromosome-positive cells are always about 1.5–1.75 times higher than detected. The following percentages have not been corrected by this factor.

Furthermore, we aimed to gain further information about the lineage and differentiation of the infiltrating cells by combining immunohistochemical analysis with FISH simultaneously on the same tissue section. Since reagents that could indisputably identify progenitor cells at different stages of differentiation are currently not available, FISH analysis was followed by immunohistochemical staining for CD45 and CD68 (to discriminate inflammatory from non-inflammatory CD45+ /CD68+ progenitor cells) or vWF (to discriminate endothelial cells). Thus, we could identify host-derived inflammatory cells (Y-chromosome-positive, CD45+ and/or CD68+), host-derived endothelial cells (Y-chromosome-positive, vWF+), host-derived progenitor cells (Y-chromosome-positive, CD45+, CD68–), and host-derived cardiomyocytes (Y-chromosome-positive, characteristic morphology). Next we aimed to sequentially utilise these techniques to evaluate the role of progenitor cells in regeneration following myocardial infarction. Representative examples of groups of cells carrying a Y-chromosome are shown in Fig. 2(a)–(d). Since lipofuscin can mimic Y-chromosome-positive cells in the spectrum orange filter, each intranuclear orange signal was verified using the single green filter ("true" Y-chromosomes were not detectable using the green filter, "false" lipofuscin signals remained, see Fig. 3(a) and (b)).

Host-derived inflammatory and non-inflammatory cells in transplanted hearts

In order to test our hypothesis that myocardial infarction leads to enhanced infiltration and differentiation of extracardiac progenitor cells, we next evaluated the relative abundance of these host-derived cell populations in normal transplanted hearts and in transplanted hearts with myocardial infarction. In patients with myocardial infarction after heart transplantation, 24.1 ± 6.3%
(mean ± SEM) of all cells carried a Y-chromosome within the infarcted region (ranging from 8.9–39.2%) adjacent to non-infarcted tissue, compared to 5.3 ± 1.1% of transplanted control patients without infarction (ranging from 2.5–12.1%, P < 0.01; Fig. 4(a)). The percentage of inflammatory (CD45+ and/or CD68+) cells of host origin found in infarcted allografts was 17.5 ± 4.6% (6.6–28.1%) and in non-infarcted allografts, 3.6 ± 1.0% (0.8–9.8%,
In patients with myocardial infarction, 5.9 ± 2.3% of all cells in the infarcted region adjacent to non-infarcted tissue were non-inflammatory CD45<sup>−</sup>/CD68<sup>−</sup> progenitor cells (2.0–13.3%) compared to 1.7 ± 0.1% in non-infarcted patients (1.1–2.4%, \(P < 0.05\); Fig. 4(c)).

Endothelial cells carrying a Y-chromosome could be detected in the arterioles of all allografts of patients with myocardial infarction at a mean level of 0.56 ± 0.26%, whereas in patients without myocardial infarction the mean level was 0.01 ± 0.01% and these cells could be found in only 1 of 9 patients (\(P < 0.05\); Fig. 4(d)). The frequencies of Y-chromosome-positive cells of each patient are presented in Tables 1 and 2.

**Host-derived cardiomyocytes in transplanted hearts**

Of 9 patients without myocardial infarction, only 2 showed Y-chromosome-positive cells in the allograft that fulfilled the criteria for host-derived cardiomyocytes. Percentages were at a rather low level of 0.1 and 0.3% respectively, with a mean of 0.04 ± 0.03% for all patients of this group. Among the 5 patients who had developed myocardial infarction after heart transplantation, Y-chromosome-positive cells fulfilling the criteria for cardiomyocytes of recipient origin could be detected in three patients, ranging from 0.2% to 0.5% with a mean percentage of 0.21 ± 0.10% among all patients of this group (Fig. 4(e)). The difference between patients with and without myocardial infarction did not reach statistical significance (\(P = 0.0548\)).

There was no correlation between the percentage of cardiomyocytes in the allograft and the time of survival after heart transplantation in either the group with myocardial infarction or the group without myocardial infarction.

**Three-dimensional confocal microscopy analysis of seemingly Y-chromosome-positive cardiomyocytes**

We proposed to evaluate the likelihood that a nucleus with a Y-chromosome is located outside but overlapping a cardiomyocyte in such a way that the examiner is misled into classifying this cell as a host-derived cardiomyocyte. Therefore, 10 cells fulfilling the criteria for cardiomyocytes using fluorescence microscopy were studied by three-dimensional confocal analysis. Interestingly, in 8 cells we found that the Y-chromosome-positive nuclei were located outside the cardiomyocyte. Two Y-chromosome-positive cells classified by fluorescence microscopy as cardiomyocytes are shown in Figs. 5(a) and (b). Three-dimensional confocal microscopy revealed that in Fig. 5(a) the nucleus carrying the Y-chromosome is not located within the cytoplasm of the cardiomyocyte, which is underneath the nucleus (video 1). This nucleus may therefore belong to a non-inflammatory progenitor cell. Analysis of the cell shown in Fig. 5(b) by confocal microscopy, however, confirmed that the nucleus with the Y-chromosome is located within the cytoplasm of the cardiomyocyte, suggesting that this cell is indeed a host-derived cardiomyocyte (video 2).
In summary, we provide evidence that thorough identification of inflammatory cells and improvement in imaging technique to minimise artefacts due to overlapping cells resulted in stepwise reduction of cells that could be identified as "host-derived cardiomyocytes" (Table 3). Thus, our data suggest that the above-mentioned low percentages of host-derived cardiomyocytes based on fluorescence microscopy are still an overestimation of the true number of cardiomyocyte differentiation.

Discussion

Regeneration of cardiomyocytes has long been thought not to occur in human heart. Recent studies in transplanted human hearts and in patients after bone marrow transplantation, however, have indicated that cardiomyocyte repopulation by extracardiac progenitors may take place.1-4

Sex-mismatched cardiac transplantation, in which a male patient receives a female donor heart, offers an exceptional opportunity to investigate stem cell-derived myocardial repopulation because the detection of the Y-chromosome clearly identifies cells of recipient origin. Using this approach, previous studies suggested that host-derived progenitor cells could regenerate cardiomyocytes. However, very different levels of host-derived cardiomyocytes in sex-mismatched transplanted patients have been described: Glaser et al.14 and Hruban et al.15 found no host-derived cardiomyocytes. Lafamme et al.1 and Muller et al.2 both described similarly low levels, 0.04% and 0.16%, respectively. Quaini et al.,1 however, found up to 15% of chimerism in cardiomyocytes. In

**Table 3** Improvement of methodology results in stepwise reduction of "host-derived cardiomyocytes"

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Percentage of host-derived cardiomyocytes (%)</th>
<th>Percentage corrected for FISH efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) FISH + morphology (Y⁺):</td>
<td>2 (n = 1)</td>
<td>3.5</td>
</tr>
<tr>
<td>(b) + immunostaining (Y⁺ /CD45⁻):</td>
<td>0.08 (n = 3)</td>
<td>0.14</td>
</tr>
<tr>
<td>(c) + immunostaining (Y⁺ /CD45⁻ /CD68⁻):</td>
<td>0.05 (n = 8)</td>
<td>0.09</td>
</tr>
<tr>
<td>(d) + 3-dimensional confocal analysis:</td>
<td>0.01 (n = 10)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The table summarises the correlation of detection method and the number of cells which were accounted for as host-derived cardiomyocytes. Each step to improve the methodology resulted in stepwise reduction of host-derived cardiomyocytes as compared to Y-chromosome detection by FISH associated with morphological criteria for cardiomyocytes alone. (a) Evaluating "host-derived cardiomyocytes" by the presence of a Y chromosome within a cardiomyocyte by conventional fluorescence microscopy, 2% of all cells were accounted for as "host-derived cardiomyocytes". (b) Additional immunofluorescence staining for CD45 significantly decreased the number of cells which were identified as "host-derived cardiomyocytes", as the majority of cells which were identified as "host-derived cardiomyocytes" by FISH alone, proved be to be false-positive, since they were CD45⁻ - inflammatory cells (with a Y-chromosome) that were overlapping with cardiomyocytes. (c) Additional staining with CD68, another marker for inflammatory cells, further decreased the percentage of cells, which were accounted for as host-derived cardiomyocytes, because more Y-chromosome-positive nuclei could be associated with overlapping inflammatory cells. (d) Additional 3-dimensional confocal microscopy revealed that 8 out of 10 host-derived cardiomyocytes had been false-positive by demonstrating that Y-chromosome-positive non-inflammatory cells had been overlapping with cardiomyocytes.

Fig. 5 3D confocal microscopy. Examples of cardiomyocytes apparently containing a Y-chromosome in the same focal plane by two-dimensional confocal microscopy. Nuclei are stained green with SYBR Green. Arrows indicate the Y-chromosome, arrowheads show autofluorescence of lipofuscin. Three-dimensional analysis of Panel a reveals that the nucleus containing the Y-chromosome is not located within the cardiomyocyte (video 1), whereas in Panel b three-dimensional analysis verifies that the cardiomyocyte contains a Y-chromosome (video 2). Scale bars = 10 μm. Videos can be viewed online (doi:10.1016/j.ehj.2004.01.017).
female patients who had undergone sex-mismatched bone marrow transplantation, Deb et al. found 0.23% of Y-chromosome-positive cardiomyocytes.

In our studies, we undertook a novel multi-step approach to identify stem cell-derived cardiomyocytes: (1) we performed FISH analysis simultaneously with immunofluorescence microscopy detection of CD45 and CD68, (2) we pre-defined morphologic criteria for cardiomyocytes which were validated by immunofluorescence with cardiac-specific antibodies, and (3) we performed three-dimensional confocal microscopy to indisputably assign nuclei to their cytoplasm.

In our studies, it turned out that many of the cells classified as host-derived cardiomyocytes based exclusively on the detection of a Y-chromosome and cardiac-specific markers (as done in previous studies), then showed a green margin around the nucleus after FITC-staining of CD45 and CD68, indicating that a leukocyte or macrophage was overlapping a cardiomyocyte (even though they appeared in the same focal plane). While the margins of cardiomyocytes are clearly visible without special staining, due to their autofluorescence, we would have been misled into classifying many more cells as Y-chromosome-positive cardiomyocytes if we had analysed them before applying antibodies against CD45 and CD68. The use of the 2-μm sections is optimised to quantify large numbers of cells with a minimum of false positive results by conventional microscopy. In initial studies we used thicker sections (5 μm). The sensitivity to detect Y-chromosomes was even lower, since the probe could not penetrate the entire section. Additionally, the problem of false positive cells due to overlapping nuclei when screening the samples with conventional microscopy was substantially worse. This problem of overlapping nuclei may in part account for the different levels of chimerism found by us and Quaini et al. Especially, the samples used by Quaini et al. were likely to have more inflammatory cells than ours, since we excluded patients with severe rejection, whereas most of the patients in Quaini’s study had died of graft failure or other inflammatory causes. Most patients in our study died from cardiogenic shock, but some also died from other causes, like haemorrhagic shock, liver failure, multi-organ failure and septic shock. Even though cardiogenic shock may have an impact on the quantity and vitality of the stem cell pool in the bone marrow, it is unlikely that potential cardiac regeneration by bone marrow stem cells prior to this event was affected in this study. However, it cannot be excluded that treatment with immunosuppressive drugs in transplanted patients could alter the physiological behaviour of bone marrow stem cells.

While the combination of FISH and immunostaining for inflammatory cells was sufficient to prevent false positive results caused by overlapping inflammatory cells, it could not exclude overlapping host-derived non-inflammatory cells (such as undifferentiated progenitor cells) as a source of false positive results. We addressed this problem by using three-dimensional confocal microscopy. Our results demonstrate that 80% of the cells that were defined as host-derived cardiomyocytes by fluorescence microscopy were false positive because the Y-chromosome-positive nucleus was located outside the cardiomyocyte. Even two-dimensional confocal microscopy can be associated with false-positive host-derived cardiomyocytes, since the plane is usually thicker than a Y-chromosome by confocal microscopy. If the plane is set to the thickness of a Y chromosome, multiple planes must be scanned through each nucleus, which is identical to the scanning process of 3-D confocal microscopy. Thus, ideally, each cell in a tissue should be analysed by 3-dimensional confocal microscopy. However, this technique is associated with extensive exposure of the tissue to fluorescent light, and fading of the fluorescent dyes could not be overcome with currently available reagents. Since such high throughput analysis is technically not feasible with the current technology, we think that our approach of stepwise combination of conventional fluorescence microscopy (to screen tissues) and 3-dimensional confocal microscopy (for further analysis of cells of interest) is an effective compromise. In summary, our results demonstrate that our multi-step approach of FISH/immunostaining for inflammatory cells and additional use of 3-dimensional confocal microscopy decreased the number of host-derived cardiomyocytes substantially. By utilising this method, only two cells out of a total of about 20,000 cells fulfilled all the criteria as host-derived cardiomyocytes. One of these two cells however, was detected in a patient who died 5 h after transplantation. Since it appears unlikely that this cell differentiated within 5 h into a cardiomyocyte, additional studies are needed to rule out the possibility that even the few cells we found were due to other causes of chimerism. In this regard, a recent study reported that a previous pregnancy with a son or receiving a blood transfusion from a female donor could lead to chimerism in the female heart.16 Also, detailed genetic analysis of these cells should be performed in order to clarify whether direct conversion of host-derived cells into cardiomyocytes and/or endothelial cells takes place, or whether this phenotype is adopted by spontaneous cell fusion as has been suggested by Terada et al. and Ying et al. In summary, these results suggest that there might be even fewer host-derived cardiomyocytes if more advanced technology becomes available. However, since all these studies were dependent on the presence of a Y-chromosome, the possibility of a resident cardiac stem cell population cannot be excluded.

Even though we found a very low percentage of host-derived cardiomyocytes in normal transplanted hearts, we speculated that repopulation may be more frequent in areas of myocardial infarction. This hypothesis was derived from animal studies, which demonstrated that freshly infarcted myocardium attracts bone marrow stem cells injected into the heart and that a rapid differentiation into cardiomyocytes occurs.7 Orlic et al. also demonstrated that if mice were treated with stem cell factor and granulocyte colony-stimulating factor (G-CSF) after myocardial infarction, regeneration of functional healthy myocardium could be observed. Because several cytokines, including G-CSF, are increased in the human heart after myocardial infarction, especially in infarcted regions adjacent to non-infarcted tissue, the
number of cardiomyocytes derived from extracardiac stem cells might be increased in humans.

Our results show for the first time that not only inflammatory cells are attracted by infarcted myocardium but also non-inflammatory host-derived progenitor cells, possibly from the bone marrow. Within 2 and 4 weeks after infarction, almost 6% of all cells can be identified as invaded non-inflammatory cells and the frequency of this process is significantly higher in infarcted compared to non-infarcted hearts. This group of Y-chromosome-positive, CD45/C0, CD68/C0 cells includes 3 subpopulations: endothelial cells, cardiomyocytes, and the remaining cells, which have not been characterised further. However, there are not many cell types left to fall within this group except for fibroblasts and mesenchymal progenitor cells. While reagents that can indisputably identify human mesenchymal stem cells in paraffin-embedded tissue are currently not available, the roundish morphology of these cells suggests that this entity represents progenitor cells rather than fibroblasts. In this regard, it has been shown that mesenchymal stem cells have the capacity to differentiate in vitro into many different lineages, such as skeletal muscle, neuronal cells, chondrocytes, adipocytes, and osteocytes, and that differentiation of these cells into cardiomyocytes occurs when they are injected into mouse myocardium.

In transplanted patients, differentiation of endothelial cells from extracardiac progenitor cells is significantly enhanced by myocardial infarction. However, only very few cells seem to differentiate into cardiomyocytes in both infarcted and non-infarcted hearts. Also, the frequency with which stem cell-derived cardiomyocyte differentiation occurs is not significantly higher in the infarcted heart, although the number of nuclei counted was limited in some of these samples because we restricted our analysis to the area directly adjacent to the infarcted area in order to focus strictly on the region of interest. Overall, these findings suggest that myocyte differentiation from endogenous stem cells is not increased in the infarcted heart after 4 weeks, and refute our hypothesis derived from animal studies.

Even though it seems reasonable to assume that more stem cells differentiate into cardiomyocytes after a longer period of time, evidence is increasing that this is not the case. Quaini et al.1 found the highest levels of chimerism in cardiomyocytes, arterioles, and capillaries soon (between 4 and 28 days) after transplantation. Conversely, the lowest levels of chimerism in these cells were detected between 396 and 552 days after transplantation. Also, in our study the level of chimerism did not increase after longer periods of time. In this regard, a recent study has demonstrated that only mesenchymal stem cells that had been transfected with Akt to overcome the viability of unaltered stem cells lead to cardiac repair in a mouse model of myocardial infarction. Because of the small number of patients, however, neither we nor other groups could study the exact correlation between the time from transplantation and infarction to death and the level of chimerism.

In summary, the present results indicate that myocardial infarction is a stimulus for the attraction and invasion of extracardiac progenitor cells. A minor portion of these cells differentiate into endothelial cells and even fewer differentiate into cardiomyocytes. Additional studies are required to identify factors that attract progenitor cells to infarcted myocardium. Furthermore, factors should be identified that promote the differentiation of these cells or that keep these cells from differentiating into cardiomyocytes. Finally, we should aim to further characterise the population of CD45/C0, CD68/C0 progenitor cells in order to identify the subpopulation that is capable of differentiating into cardiomyocytes. While physiologic regeneration of the heart seems insufficient, this might provide valuable insights for generating cardiomyocytes in vitro to be used for future cellular therapy.

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References


