Cardiomyocytes of Noncardiac Origin in Myocardial Biopsies of Human Transplanted Hearts

Patrick Müller, MD; Peter Pfeiffer, MS; Jörg Koglin, MD; Hans-Joachim Schäfers, MD; Ute Seeland, MD; Ingrid Janzen, MD; Steffi Urbatsch, PhD; Michael Böhm, MD

Background—Cell replacement therapy with stem cells able to differentiate into cardiomyocytes has been discussed as a method for remodeling damaged myocardium. A physiological or pathophysiological situation in which this phenomenon might be relevant is not known. We studied the origin of cardiomyocytes in myocardial biopsies of male patients that had undergone sex-mismatched cardiac transplantation to determine whether cells containing a Y chromosome (and therefore being of recipient origin) are able to differentiate into cardiomyocytes.

Methods and Results—Myocardial biopsies (n=21) were obtained from the right ventricles of male patients (n=13) who had undergone sex-mismatched heart transplantation. Tissue from 1 nontransplanted male and myocardial biopsies from sex-matched heart–transplanted patients served as controls. Cells from donor and recipient origins were identified by fluorescence in situ hybridization with the use of specific probes for X and Y chromosomes on paraffin sections of the biopsies. Cell types were identified by using immunostaining procedures on the same tissue sections. Cardiomyocytes of recipient origin were detected in 8 of 13 male recipients of female hearts. They were connected by gap junctions with adjacent myocytes. Of the cardiomyocyte nuclei, 0.16±0.04% (mean±SEM, median 0.09%) contained the Y-chromosomal marker. There was no detectable correlation with the extent or number of rejection episodes, time of transplantation, or medical treatment regimen.

Conclusions—These results show that regeneration by cells of noncardiac origin (differentiated into cardiomyocytes and physiologically linked to neighboring myocytes) can be detected even in small myocardial biopsies. This may lead to new diagnostic and therapeutic strategies in the treatment of myocardial infarction, inflammatory heart disease, and/or heart failure. (Circulation. 2002;106:31-35.)

Key Words: transplantation ■ sex ■ heart diseases ■ genes ■ cells

For the last few decades, it has been widely accepted that the cardiac muscle is a terminally differentiated organ that is not capable of regeneration and that cardiac myocytes withdraw from the cell cycle after birth. Recently, evidence of myocyte proliferation in end-stage cardiac failure and in human hearts after myocardial infarction has been provided. Because of reports of bone marrow–derived cells differentiating into cardiomyocytes in rodents, we wondered whether this process occurs in human myocardium, whether it could potentially contribute to myocardial regeneration in humans, and whether such a phenomenon could also be detected with the use of routine myocardial biopsy specimens. An ideal constellation for the study of the engraftment of cells can be found in male recipients of female donor hearts, because in these patients, cardiomyocytes of recipient (male) origin can be identified by the Y chromosome. Therefore, we examined myocardial biopsies of sex-mismatched transplanted patients by in situ hybridization for Y and partially X chromosomes to detect cardiomyocytes engrafted from cells of recipient origin. We also sought to determine whether the newly formed cells were connected by gap junctions with adjacent cardiomyocytes.

Methods

Patients

We studied 21 biopsies from 13 male heart transplant recipients who received organs from female donors. Data about underlying heart disease as a cause for transplantation, time of biopsy, patient age, rejection grade according to the International Society of Heart and Lung Transplantation, and medications are shown in the Table.

From all patients, right ventricular myocardial biopsies were taken according to the posttransplantation evaluation program. All heart specimens were fixed in 4% PBS-buffered formalin and were paraffin-embedded according to standard procedures. As positive controls, right ventricular sections from the explanted heart of a male patient with dilated cardiomyopathy and from the heart biopsy of a
male sex-matched transplanted patient were used. Negative controls included biopsies of 2 sex-matched transplanted female patients.

**Immunofluorescence Analysis**

Immunofluorescence study was performed on 6-μm-thick sections by applying monoclonal antibodies against α-sarcomeric actin (clone 5c5, Sigma Chemical Co) and myoglobin (Sigma) to detect cardiomyocytes. Serial sections were also counterstained for endothelial cells (von Willebrand factor, clone F8/68, DAKO), smooth muscle cells (anti-α-smooth muscle actin, clone 1A4, Sigma), leukocyte common antigen (CD45RB, DAKO), and macrophages (CD68, clone KP1, DAKO). Immunostaining for connexin43 (Sigma) was used to detect intercellular connections and electrical coupling between the cardiomyocytes. Fluorescein isothiocyanate–conjugated anti-mouse IgM, anti-mouse IgG, and anti-rabbit IgG were used as secondary antibodies (1:25, Dianova). The slices were microwave-treated at 560 W for 5 minutes and then incubated for 1 hour at 37°C with the first antibody, followed by the appropriate secondary antibody for 45 minutes.

Because digestion with proteinase K (see below) obliterated antigenic sites for α-sarcomeric actin antibody binding, these sections were counterstained immediately after cardiomyocyte immunostaining with Vectorshield Mounting Medium with DAPI (Vector Laboratories) for detecting nuclei and then systematically and thoroughly photographed at a magnification of ×400. The photographs were later compared with photographs taken after fluorescence in situ hybridization (FISH) to identify cardiomyocyte nuclei.

**Fluorescence In Situ Hybridization**

After immunostaining, FISH was performed as described elsewhere, with only minor modifications. Pretreated slices were digested with proteinase K (2 g/mL, Sigma) for 5 minutes. The presence of chromosome X was detected with the use of the biotinylated α-satellite DNA probe DXZ1 (Q-Biogene), and the presence of chromosome Y was detected by DNA probe Y3.4 (American Type Culture Collection, vector pBR322, digoxigenin-labeled with the Nick Translation System [GIBCO-BRL Life Technologies]).

The biotin-labeled probe DXZ1 was detected by fluorescein streptavidin (Vector Laboratories) and amplified with the use of a biotinylated anti-streptavidin antibody (Vector Laboratories) and, again, fluorescein streptavidin. The digoxigenin-labeled probe Y3.4 was detected by anti–digoxigenin-Cy3 (Dianova).

**Tissue Analysis**

Sections stained for α-sarcomeric actin were examined at a magnification of ×400 with a Nikon E 600 microscope (Nikon Europe) equipped with filters suitable for 4′,6-diamidino-2-phenylindole and fluorescein isothiocyanate. Photographs were taken with a Nikon DXM1200 digital camera (Nikon Europe). These images were later correlated with the corresponding micrographs obtained after FISH (see below) to identify hybridization signals in cardiomyocyte nuclei. FISH signals and immunostaining other than with α-sarcomeric actin were evaluated field by field at a magnification of ×1000 with an Olympus-BX-70 epifluorescence microscope with suitable filters for 4′,6-diamidino-2-phenylindole, fluorescein isothiocyanate, and Cy3. Evaluation was performed according to the

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**Information About Patients and Biopsies**

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HTx indicates heart transplantation; ISHLT, International Society for Heart and Lung Transplantation; Cyc, cyclosporine; Tac, tacrolimus; Aza, azathioprine; Pred, prednisone; Myc, mycophenolate mofetil; ACE, ACE inhibitor; Stat, statin; Ca-A, calcium antagonist; Diu, diuretics; C nuclei, cardiomyocyte nuclei; Y-pos C nuclei, cardiomyocyte nuclei showing Y chromosome; ICM, ischemic cardiomyopathy; DCM, dilated cardiomyopathy; AMI, acute myocardial infarction; PH, pulmonary hypertension; and x, use of drug.

*More than 1 section per biopsy examined.
strict criteria of Hopman et al. Two independent microscopic examiners (P.M. and P.P.) were blinded to the donor/recipient status. To exclude the possibility of detecting overlapping nuclei of non-myocytes, only single located nuclei, totally surrounded by cardiomyocyte cytoplasm, were counted (see the Table).

In the positive controls, at least 400 nuclei were counted to examine hybridization efficiency of the Y probe. In the biopsies of male recipients of a female heart and in the negative controls, all nuclei identified as being of cardiomyocyte origin were counted. Photographs of hybridization signals were taken with the use of a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics K.K.) and specialized software (ISIS 4, Metasystems).

### Results

The biopsies of 2 sex-matched transplanted female patients, serving as negative controls, showed no Y chromosome at all in a total of 2053 of 3801 examined cardiomyocyte nuclei. Positive controls yielded a high percentage of cardiomyocytes with the Y chromosome: 66.47 ± 2.78% (mean ± SEM) of the cardiomyocyte nuclei in the nontransplanted male patient and 55% of the cardiomyocyte nuclei in the sex-matched transplanted male patient showed an Y chromosome.

In serial sections of cardiac muscles from right ventricular biopsies of female donor hearts implanted into male patients, single cardiomyocytes contained Y chromosomes (and, thus, were of male origin) in 8 of 13 sex-mismatched transplanted patients (Table). Cardiomyocyte nuclei containing a Y chromosome (see Figure 1) and, therefore, being of recipient (male) origin were located as single cells and were not found to occur in clusters. Of the 31 787 cardiomyocytes studied, 0.16 ± 0.04% (mean ± SEM, median 0.09%) were of recipient origin (for details, see the Table). The numbers of examined cardiomyocyte nuclei ranged from 73 to 2387 per biopsy. Biopsy samples containing the Y chromosome tended to be bigger and, therefore, to contain more nuclei per section (range 598 to 2387 nuclei versus 73 to 664 nuclei in sections of negative-biopsy specimens). In 6 patients, we were able to examine biopsies obtained at 2 different time points (Table). A significant increase of recipient-derived cells over time was not detected. In 5 of these patients, both biopsies contained cardiomyocyte nuclei with a Y chromosome. In 1 case, the first biopsy was negative, and the second was positive. For 1 patient, we sampled 3 small biopsies from different time points, which were all negative.

The newly formed cardiomyocytes formed intercellular connections with the surrounding myocardium; these were shown by immunostaining with a connexin43 antibody (see Figure 2).

Nuclei containing a Y chromosome were also found in endothelial (see Figure 2) and smooth muscle cells (but at a higher frequency than in cardiomyocytes) and even in biopsies not showing fluorescence signals for the Y chromosome in cardiomyocytes. Because there were only small numbers of endothelial and smooth muscle cells in the myocardial biopsies and because endothelial nuclei often overlapped, quantification was not reliable and, therefore, not performed. Macrophages and leukocytes were in the majority for cells of recipient origin (see Figure 2).

### Discussion

Recently, there has been increasing evidence that adult cells have far greater plasticity than previously thought, with bone marrow–derived cells turning into skeletal muscle,9 liver,10 brain,11 endothelium,12,13 and even cardiac muscle.3–5,14 Although the findings of cardiomyocytes formed from bone marrow in rodents have been suggestive, it has been impossible to extrapolate these findings to human physiology without studying human tissues directly. Using the therapeutic “experiment” of sex-mismatched heart transplantation, we conclude that the human adult heart has the power to regenerate cardiomyocytes by recruiting cells of noncardiac origin.

The newly formed cardiomyocytes were intercellularly connected and, therefore, probably electrically coupled with other cardiomyocytes, as shown by immunostaining for connexin43, which is an integral membrane protein constituent of gap junctions.15
Numbers of newly formed cardiomyocytes attained a mean±SEM of 0.16±0.04% with a median of 0.09%, but the real percentage might have been underestimated. Because of hybridization efficiency and partial sampling of nuclei in the 6-μm thin tissue sections, the Y-chromosome probe in positive controls was detected in only 66.47±2.78% of the cardiomyocyte nuclei in the patient with dilated cardiomyopathy and in 55% of the cardiomyocyte nuclei in the sex-matched transplanted male patient. Therefore, one might speculate that replacement would be more pronounced when cardiac injury is more severe. A recently published study by Quaini et al,16 who examined sex-mismatched transplanted hearts after the subjects had died, has shown higher numbers of cardiac chimerism, although our hybridization efficiency was comparable. But in another postmortem study,17 prevalence of newly built cardiomyocytes was also very low (0.04%). One reason for the difference between the study of Quaini et al and our findings may be that we used very strict criteria8 in our fluorescence microscopic setting to define nuclei as being of cardiomyocyte origin: We counted nuclei only if they were surrounded totally by myocytic cytoplasm and if there was no other nucleus visible in the same cell. In addition, none of our patients had episodes of severe rejection, whereas most patients in the study of Quaini et al had died because of graft failure or other inflammatory causes, which may have contributed to their higher numbers. So our cases may more likely represent the real regeneration status in the living. But taking into account the fact that Laflamme et al17 also found very low numbers in their autopic cases, we found that to be a rather unsatisfactory explanation. In the end, the difference may also be due to the fact that the interval between transplantation and death was much shorter in the study of Quaini et al than the interval between transplantation and biopsy in our patient group and in the patient group of Laflamme et al. So one mechanism could be that after transplantation, a rather high number of new cells showing signs of cardiomyocyte differentiation are built, but they die rather quickly. On the other hand, it is imaginable that the patients of Quaini et al had rather high rates of inflammatory cells in their newly transplanted hearts, so that many of the Y-positive nuclei belonged to overlapping nuclei of leukocytic cells than to real cardiomyocytes.

Biopsy samples showing no Y chromosome tended to be smaller and to contain fewer nuclei. Therefore, one cannot exclude the possibility that an impact of a low number of nuclei might also be responsible for an underestimation of regenerated cells in human transplanted hearts. However, these limitations would lead to an underestimation but not to an overestimation of regenerated myocardial cells. While interpreting the presented data, one must also keep in mind that samples were from transplanted patients who had all suffered more or less from rejection episodes. This may have represented a stimulating factor for the recruitment of stem cells. So the numbers of regenerated cardiomyocytes may be larger in transplanted patients than in people not suffering from transplant rejection (such as healthy persons). On the other hand, the number of regenerated myocytes may be comparable or even higher in patients suffering from myocardial infarction or dilated cardiomyopathy, in which mitotic index and expression of Ki-67 (a nuclear protein associated with cell proliferation) as a sign of cell division have been shown to be increased.1,2 In line with a significant regeneration in diseased hearts is the finding that the number of cardiomyocytes increases rather than decreases with hyper trophy in congestive heart disease.18

Other authors detected proliferating myocytes expressing Ki-67 after myocardial infarction in mice and reported that these mitotic active cells were derived either from resident cardiomyocytes or from circulating stem cells.4 Our results indicate the existence of noncardiac cells that are able to differentiate into cardiomyocytes, because the detected Y chromosome-containing cells could be derived only from the host organism and not from resident cells of the donor heart. This finding is supported by another study,19 in which mice after myocardial infarction were treated with stem cell factor and granulocyte colony-stimulating factor. These animals showed the production of functional healthy myocardium after receiving this form of therapy. A similar approach,
raising numbers of immigrating and engrafting cells to build significant new cardiac muscle mass, could be undertaken in humans. It would be an interesting alternative or at least an addition to the transplantation of myoblasts, fetal cardiomyocytes, or stem cells into damaged hearts as proposed by others.\textsuperscript{6,20–22} The present study provides evidence for the applicability of this principle.

In summary, these results challenge the dogma that the heart is not able to regenerate. Recruitment of noncardiogenic cells may be a component of the growth reserve of the human heart. Although the percentage of regenerated cardiomyocytes is rather low, the mechanism could be used to replace damaged myocardium by raising the numbers and by promoting the migration of the respective precursor cells into damaged myocardium. Such a therapeutic approach could be an interesting alternative to the transplantation of myoblasts, fetal cardiomyocytes, or stem cells. But because of the low numbers of newly generated cells in transplanted patients, further studies are necessary to determine whether it is possible to regenerate enough cardiomyocytes to significantly enhance the pump function of a damaged heart. Because the newly built cardiomyocytes can be detected in diagnostic biopsies, the amount of cellular reparation could be a marker of precedent damages imposed on the heart, therefore acting like a cardiac memory.

Acknowledgments

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References

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