The demonstration of the presence of dividing primitive cells in damaged hearts has sparked increased interest about myocardium regenerative processes. We examined the rate and the differentiation of in vitro cultured resident cardiac primitive cells obtained from pathological and normal human hearts in order to evaluate the activation of progenitors and precursors of cardiac cell lineages in post-ischemic human hearts. The precursors and progenitors of cardiomyocyte, smooth muscle and endothelial lineage were identified by immunocytochemistry and the expression of characteristic markers was studied by western blot and RT-PCR. The amount of proteins characteristic for cardiac cells (α-SA and MHC, VEGFR-2 and FVIII, SMA for the precursors of cardiomyocytes, endothelial and smooth muscle cells, respectively) inclines toward an increase in both α-SA and MHC. The increased levels of FVIII and VEGFR2 are statistically significant, suggesting an important re-activation of neoangio genesis. At the same time, the augmented expression of mRNA for Nkx 2.5, the transcriptional factor for cardiomyocyte differentiation, confirms the persistence of differentiative processes in terminally injured hearts.

Our study would appear to confirm the activation of human heart regeneration potential in pathological conditions and the ability of its primitive cells to maintain their proliferative capability in vitro. The cardiac cell isolation method we used could be useful in the future for studying modifications to the microenvironment that positively influence cardiac primitive cell differentiation or inhibit, or retard, the pathological remodeling and functional degradation of the heart.

Key words: cardiac cell lineages in vitro cultures resident primitive cells.

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Recently, the presence of dividing primitive cells has been observed in organs considered to be terminally differentiated, such as brain (Uchida et al. 2000) and heart (Beltrami et al. 2001, 2003). Lineage negative, c-kit positive or Sca-1 positive cells isolated from murine and dog hearts have been demonstrated to produce cardiomyocytes, smooth muscle and endothelial cells in vitro (Rossi et al. 2005, Linke et al. 2005, Orlic et al. 2001). Moreover, the precursors and progenitors of all cardiac cell lineages have been identified on the basis of the expression of specific markers: transcription factors MEF-2C, GATA-6 and Ets-1 for progenitors and the cytoplasmic proteins α-Sarcomeric Actin (αSA) or myosin, Smooth Muscle Actin (SMA) and Factor VIII (FVIII) for the precursors of cardiomyocytes, smooth muscle and endothelial cells, respectively (Urbanek et al. 2005).

These observations have prompted studies focusing on the regeneration of tissue damaged by diseases (Urbanek et al. 2003) or the aging process (Anversa et al. 2005), as the cardiac-resident primitive cells seem to be a promising target for acute and chronic heart disease therapy in which cardiac regeneration may be accomplished by enhancing the normal turnover of myocardial cells (Urbanek et al. 2005, Oh et al. 2003, Murry et al. 2004). On the other hand, cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts, being the more representative of the cell populations in the myocardium, are damaged in a different way after ischemia, with fibroblasts in particular being involved in the pathological remodeling of the injured heart leading to the heart transplantation. At the same time, recent studies have shown that myocytes proliferation in adult human hearts may be stimulated and modulated by several factors (Postiglione et al. 2006, Urbanek et al. 2005). The present study was designed to establish whether cardiac-resident primitive cell populations exhibit different rate of
differentiation in normal and in pathological hearts.

Our objective was to evaluate the capability of the human heart to induce an increase in the number of resident cells able to differentiate in the cell lineages that are functionally most important, in other words cardiomyocytes, smooth muscle and endothelial cells. To this end we established a method to isolate and co-culture the progenitors and precursors of cardiac cell lineages from human biopsy specimens. The quantification of cardiac primitive cells of cardiomyocyte, smooth muscle and endothelial lineage obtained from normal hearts and hearts with post-ischemic cardiomyopathy was performed in vitro, as was the observation of the expression level of cytoplasmic proteins characteristic for the differentiated cardiac cells. The technique of cell culturing used in this study could well have an impact on the study of the interactions between cardiac cell populations.

Materials and Methods

Cell cultures

Cardiac primitive cells were isolated from bioptic fragments (1,1-2.6 mm$^2$) of the right ventricle of normal and pathological human hearts. The first were obtained from healthy donor hearts before transplantation (n=10, ranging age 16-58, death caused by trauma or cerebral hemorrhage). Similar biopsies were taken from the pathological hearts within 4 hours of their explantation, as a result of the terminal heart failure associated with post-ischemic dilative cardiomyopathy (n=10, age range 45-58, Left Ventricular Ejection Fraction 15-27%, NYHA class III/IV, duration of heart failure 5-13 years). All samples were obtained with the informed consent of the patients in accordance with the requirements of the hospital ethics committee. The investigation conforms to the principles outlined in the Declaration of Helsinki.

All the tissue specimens were washed in the sterile PBS solution and put on the glass-covered culture dishes in the F12K medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum and 5% horse serum (Invitrogen, Carlsbad, USA), 5 ng/mL bFGF (Peprotech, London, UK), 0.2 mM glutathione (Sigma-Aldrich), Penicillin G 50,000 U and streptomycin 50 mg (Invitrogen). The outgrowth of cells from the bioptic fragments was inspected at time periods ranging from 2 to 4 weeks. Once the adherent cells were more than 75% confluent, they were detached with 0.25% trypsin-EDTA (Sigma-Aldrich): the cover glasses and the biotic fragments were then removed and the cells were replated using a 1:4 dilution.

All morphological observations were performed when the cells were replated for the first and the third time (P1 and P3); biomolecular studies in the form of western blot and RT-PCR were performed at P3.

Growth kinetics

To determine in vitro growth kinetics of cells derived from normal and pathological hearts, the cells were seeded at the density of 2×10$^4$/cm². The cells were trypsinized and counted on a hemocytometer after 2, 4, 6 and 8 days.

BrdU incorporation

BrdU was added to each culture dish in the exponential phase of the growth curve to give a final concentration of 10 µM and incubated at 37°C for 1 hour. Detection of incorporated BrdU was performed following the supplier’s protocol (Roche Diagnostics GmbH, Germany). In brief, this involved removing the medium and fixing the cells with ethanol for 20 minutes at -20°C. The cells were subsequently covered with anti-BrdU antibody diluted 1:10 in incubation buffer and incubated for 30 minutes at 37°C then placed in anti-mouse-Ig-fluorescein solution for a further 30 minutes at 37°C. The dishes were examined under a fluorescence microscope.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes. They were then covered for 30 minutes with 10% serum derived from the species in which the secondary antibody was raised, and subsequently incubated with primary antibody for 60 minutes at 37°C. We used antibodies against c-kit (Dako Cytomation, Glostrup, Denmark), MDR-1, GATA-6, (Santa Cruz Biotechnology, Santa Cruz, USA), α-SA, SMA, FVIII, fibronectin (Sigma-Aldrich), ventricular MHC (Chemicon, Temecula, USA) and VEGF-R2 (Flk-1/KDR, Novocastra, Newcastle, UK). After PBS washes, rhodamine or fluorescein conjugated anti-rabbit or anti-mouse IgG antibody was added to the cells for 60 minutes at 37°C. The nuclei of cells
were labeled with DAPI for 15 minutes at room temperature before the final washes in PBS and were then mounted in Vectashield. Signals were visualized with a Leica DMLB fluorescent microscope.

**Western blotting analysis**

Protein extracts were prepared from the cultures of cells derived from normal and pathological hearts. In brief, the samples were incubated in a lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl and 0.1% Triton X-100) supplemented with proteases inhibitors (leupeptin 10 µg/mL, pepstatin A 10 µg/mL, PMSF 0.4 mg/mL and EDTA 5 mg/mL) for 30 minutes and centrifuged at 14,000 µg for 20 minutes. The BioRad Protein Assay was used to determine the supernatant protein concentration. Proteins (80 µg) were loaded on a 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto nitrocellulose membrane by semi-dry electro-blotting. The membranes were blocked with 5% (w/v) non-fat milk for 3 hours and incubated overnight in the blocking solution with one of the following primary antibodies: α-SA (Sigma-Aldrich), ventricular MHC (Chemicon), ventricular MLC (Abcam, Cambridge, UK), VEGF-R2 and FVIII (Sigma-Aldrich). After washing, the membranes were probed with an HRP-labeled secondary IgG in 3% non-fat milk for 1 hour. Finally, bands were visualized by chemiluminescence (Amersham, Buckinghamshire, UK) followed by autoradiography.

**Semi quantitative RT-PCR**

Total RNA was isolated by lysing the fresh cells in Trizol solution (GIBCO BRL, Life Technologies, Rockville, USA) according to the supplier’s protocol. The yield and integrity of each RNA sample were checked by spectrophotometrical measurement of A260 and agarose gel electrophoresis, respectively. 5 mg of total RNA from each sample were reverse-transcribed by using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Arlington Heights, USA) according to the protocol supplied by the manufacturer. The pd(N)6 primer provided in the kit was used.

The same volume (2-4 mL) of Reverse Transcription Reaction from each sample was used for subsequent PCR amplification with the primer sets prepared for the target genes and the glyceraldehyde phosphate dehydrogenase (GAPDH) housekeeping gene.

Samples without cDNA served as negative controls for PCR amplifications.

The primers used for PCR and the expected size of their amplification products are summarized in Table 1 Specific primers for FVIII, Nkx2.5 and GATA6 were designed with the assistance of Oligo4 software. Primers for GAPDH (Arcari et al. 1983) were gently gifted by Prof. Arcari.

Primers for VEGF-R2 were designed according to the literature (Enomoto et al. 2003).

The amplified products were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination.

The levels of target cDNA were estimated by densitometric scanning and normalized against GAPDH loading controls. Densitometric analyses of the PCR products were performed with NIH Image software version 1.62F.

**Statistical analysis**

Summary values are given as the mean ± standard deviation of the mean. The unpaired Student’s t-test was used for statistical analysis. Data were considered to be statistically significant at a value of p<0.05.

**Results**

**Growth kinetics**

**Growth curves.** Cells from pathological hearts always showed a higher growth rate when compared with cells from normal hearts. The increase appeared more significant at P3, when the proliferation rhythm of cells from explanted hearts was ten

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Primer</th>
<th>Sequences</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5’-CACCATCTTCCAGGAGCGAG-3’</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TCACGCCACAGTTTCCCGGA-3’</td>
<td></td>
</tr>
<tr>
<td>GATA-6</td>
<td>Forward</td>
<td>5’-GCCCTCCTCACGAGGCAGA-3’</td>
<td>378</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TCTCCCGCACCAGTCATCACC-3’</td>
<td></td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>Forward</td>
<td>5’-GATGTTGCTCTGAGTCGCCTC-3’</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GCTGGGCTCTGTCCTGTCCTG-3’</td>
<td></td>
</tr>
<tr>
<td>FVIII</td>
<td>Forward</td>
<td>5’-CAGCTCTCATCACGAGTCGTTC-3’</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ATGCGAGATTGCTGCAAATG-3’</td>
<td></td>
</tr>
<tr>
<td>Nkx 2.5</td>
<td>Forward</td>
<td>5’-CTCCACAGCCTCCCTGAC-3’</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CTATTGGCAAGCTGAT-3’</td>
<td></td>
</tr>
</tbody>
</table>
times higher than that of normal cells (Figure 1). BrdU assays. Quantitative determination of DNA synthesis made by BrdU showed a significant increase of newly synthesized DNA in cell cultures from post-ischemic hearts over that of cell cultures from normal hearts, with a progressive decrease depending on the culture passages (Table 2).

**Characterization of the cultured cells**

Morphology and number of primitive, progenitor and precursor cells: immunocytochemistry.

Immunodetection at culture passages P1 and P3 showed that cells derived from biotic pieces of normal and pathological hearts had membrane, cytoplasmic and nuclear antigens characteristic for the primitive cells of cardiac lineages (cardiomyocytes, endothelial and smooth muscle cells, fibroblasts). The cell percentage was determined by three independent observers by counting 30 fields in every culture dish. The number of cells that expressed one of the cited markers was then compared with the number of cell nuclei (Table 3). The immunopositivity for the different markers of cardiac primitive cells is shown in Figure 2.

**Western Blotting.** The protein characteristics for the particular populations of primitive cells derived from normal and pathological hearts were quantified by western blot. The expression of α-SA was 1.9 times higher in the cells of the hearts with post-ischemic cardiomyopathy. Similarly, MHC was expressed more strongly in these cells (1.3-fold increase). The expression of FVIII and VEGFR-2 was increased by 1.5 and 17 times higher, respectively (Figure 3).

**RT-PCR.** Using RT-PCR to quantifying the mRNA for the proteins investigated substantially con-

![Figure 1. Cell growth rate at the first (P1) and the third (P3) in vitro passages. Pathological hearts cells always showed higher growth rates than cells from normal hearts.](image)

**Table 2. BrdU incorporation in vitro (% of positive cells).**

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>From normal hearts</td>
<td>29.4±0.5</td>
<td>25.9±0.8</td>
</tr>
<tr>
<td>From pathological hearts</td>
<td>35.1±0.9</td>
<td>30.6±0.5</td>
</tr>
</tbody>
</table>

**Table 3. Immunocytochemical characterization of cells in vitro (%).**

<table>
<thead>
<tr>
<th>Marker positivity</th>
<th>Cell lineage</th>
<th>From normal hearts</th>
<th>From post-ischemic hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
<td>P3</td>
<td>P1</td>
</tr>
<tr>
<td>c-kit/mdr-1</td>
<td>Primitive cells</td>
<td>1.85±0.12</td>
<td>1.77±0.1</td>
</tr>
<tr>
<td>α-SA/MHC</td>
<td>Cardiomyocytes</td>
<td>4.93±0.2</td>
<td>4.94±0.4</td>
</tr>
<tr>
<td>FVIII</td>
<td>Endothelial cells</td>
<td>2.6±0.15</td>
<td>1.3±0.09</td>
</tr>
<tr>
<td>SMA/GATA6</td>
<td>Smooth muscle cells</td>
<td>3.87±0.3</td>
<td>3.77±0.32</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Fibroblasts</td>
<td>86.7±8.0</td>
<td>88.2±7.9</td>
</tr>
</tbody>
</table>
Figure 2. Immunopositivity for different markers of cardiac primitive cultured cells. Primitive cells in A are c-kit and MDR-1 positive. In B, cardiomyocytes precursors are stained by α-sarcomeric actin and MHC antibodies; in C VEGFR-2 and factor VIII immunopositivity stain the endothelial cells. Magnification 20X (A left), 40X (B left and right, C left), 100X (A and C right).
firmed the increased number of precursors for endothelial cells, cardiomyocytes and smooth muscle cells in the cultures from pathologic hearts, with a light but constant increase in the expression of mRNA for Nkx2.5 and GATA-6, suggesting a neo-differentiation of the cardiomyocytes and smooth muscle cells precursors, and a marked increase in mRNA expression for VEGFR2, in the cells from explanted hearts as compared to the cells from normal hearts, which indicates neo-angiogenesis (Figure 4).

**Discussion**

The cell cultures obtained from the biotic fragments of normal hearts and from the pathological hearts with post-ischemic cardiomyopathy contained, as expected, a small number of stem cells, a relatively larger population of cardiac progenitors and precursors, and an extremely high population

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**Figure 3.** The expression of protein characteristic for lineage committed cardiac primitive cells in vitro was evaluated by western blot analysis of equal amounts of proteins in whole cell lysates. The results are shown as an increase of densitometric value for pathological cells normalized to normal cells. Values are means ± SD; n = 10. *p<0.05.

**Figure 4.** mRNA expression of specific markers of cardiac cell lineages in precursors and progenitors. A, Representative RT-PCR results for FVIII, VEGFR2, Nkx2.5 and GATA-6 are shown. B, The relative expression of FVIII, VEGFR2, Nkx2.5 and GATA-6 was quantified from ten independent experiments, and the mean ± SD is presented for each marker. *p<0.05.
of myofibroblasts. Of all these cells, the last group represents the cells with the highest proliferation potential.

It is well known that primitive cells represent only small fraction of the cells in any tissue. The frequency of hematopoietic stem cells has been estimated as being between one per 10,000 and one per 100,000 in bone marrow cells (Conti et al. 2005). In the mouse heart the number of the c-kit positive cells varies from 10 to 85 in 100 mm² (Beltrami et al. 2001). Their main role is to give rise to rapidly proliferating and migrating progenitors and precursors of cells of different lineages that, ultimately, undergo differentiation while retaining the capability of self-renewal. This requires a balance between the asymmetric mode of division and both the symmetrical self-renewing and commitment divisions. The mechanisms controlling their cell cycles are not well known. Many studies indicate genes and proteins responsible for the fate of stem cells are at work here, in vivo. However, these processes depend on specialized cellular microenvironments and the in vitro expansion of pure populations of adult stem cells remains elusive (Oh et al. 2003). For these reasons, it is not surprising that, in our study, the bioptic fragments of cardiac tissue 1,1-2,6 mm² in size only enabled the outgrowth of a few lineage negative, c-kit positive stem cells. From this point of view, our study of the histological sections performed on the specimens (data not shown) confirmed the conclusions drawn by other authors in their in vivo investigations of the hypertrophied myocardium of patients with chronic aortic stenosis, which showed the growth and differentiation of cardiac stem cells in mature myocytes as markedly enhanced in pathological tissue fragments (Urbanek et al. 2003).

Also worth noticing is the increase in the relative number of cardiac precursors and progenitors isolated from the explanted human hearts with chronic post-ischemic cardiomyopathy in vitro (our percentage results indicate a four-fold increase in α-SA expressing cells and an almost two-fold increase in endothelial cells). This reflects an activation of the regeneration potential of the human heart under pathological conditions and the continuation of this activation even during in vitro culturing. Moreover, although there is no direct evidence that cardiac progenitor doubling is primarily involved in the observed increase in cell proliferation, we feel it is important to report the indirect evidence that the decrease in fibronectin positive cells observed in post-ischemic hearts is statistically significant (Table 3).

We observed that the growth rate of cardiac cell populations in vitro was always higher in cultures from explanted hearts; this is not really surprising, because fibroblasts are the more representative cell population in our co-cultures and are activated by the disease. As regards the expression of proteins characteristic for cardiac cells (α-SA and MHC, VEGFR-2 and FVIII, SMA for the precursors of cardiomyocytes, endothelial and smooth muscle cells, respectively) there is a trend towards an increase in both α-SA and MHC, while the increased levels of FVIII and VEGFR2 are statistically significant.

This trend offers food for thought, as it implies an important re-activation of neoangiogenesis; on the other hand, the increased expression of mRNA for Nkx 2.5, the transcripational factor for cardiomyocyte differentiation, also suggests the persistence of differentiative processes in terminally injured hearts.

In this study, the in vitro rate of activation and differentiation of cardiac primitive cells was measured using specimens of human hearts in two extreme conditions: those with no sign of cardiovascular disease and other manifesting the cardiomyopathy associated with a terminal insufficiency, where only heart transplantation can save the life of the patient. It is not known at what time during the development of heart failure this activation occurs, or when the primitive cells' capacity for producing committed progenies and conserving the self-renewal properties is finally at an end resulting in the exhaustion of their pool in the heart.

The evidence that in vitro cultured cells from pathological hearts show a higher number of cardiac lineages positive cells than the cultures obtained from normal hearts confirms their activation in response to the pathological conditions during the progress of a disease. Further investigation is required in order to determine whether this activation, which does not cease even after all the remodeling of the heart or with the gradual worsening of its function during ischemic heart disease, leads to the depletion of the cardiac stem cell pool.

**Acknowledgements**

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References


