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The isolated beating heart as a model for stem cell transplantation studies

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Abstract

In the isolated rat heart model all types of cells and matrix elements with their interactions are represented, while external interferences by neuro-hormonal factors are excluded.
We wondered whether this model can be used to study the homing of mesenchymal bone marrow stem cells (MSCs) injected in the ventricular wall of a beating heart.
We studied the behaviour of MSCs injected in infarcted hearts. We showed that in infarcted hearts MSCs remain in the site of injection forming clusters of cells near the ischemic area.
We suggest that the isolated heart is a good model to study early features of MSC homing. Importantly. The results clearly show that infarcted myocardium influences transplanted MSCs behaviour within the beating heart.

Introduction

A wide range of stem/progenitor cell types have been used for cardiac cell therapy, myoblasts and bone marrow stem cells (Pittenger and Martin, 2004; Dimmeler et al. 2005). However, the mechanisms for progenitor cells' homing to sites of tissue injury are only understood rudimentarily. In particular, nothing is known about early (first few hours) cell survival and distribution in the receiving beating heart (Pittenger and Martin, 2004; Dimmeler et al. 2005).
We wondered whether isolated hearts could be used as a model for studying the early stages of mesenchymal stem cell (MSC) homing in infarcted myocardium.
The common methods used for stem cell homing and cell to cell interaction studies are based on *in vitro,* either alone or in co-culture with different types of cells, and *in vivo* where the stem cells are injected intravascularly or directly into the studied organ (Pittenger and Martin, 2004; Dimmeler et al., 2005). Isolated heart represent a model in which all types of cells and matrix elements with their interactions are represented, while external "disturbing" elements such as neuro-hormonal influences can be excluded or, if necessary, included under experimental control.
In this study, the isolated and perfused rat heart model was used for investigating the homing of GFP-stably-transfected adult mesenchymal bone marrow stem cells, which can be easily recognized because of their well evident autofluorescence in infarcted hearts.

Materials and methods

Isolation of stem cells and cell cultures

MSCs were harvested from the bone marrow of the femurs of GFP-stable transfected (GFP under β-actin promoter, Okabe et al., 1997) adult rats and treated in culture as described in Raimondo et al (2006). To verify the mesenchymal origin of these cell, they were stimulated with dexamethasone, insulin and IBMX for 5 days. With this treatment they differentiate in adypocites (Raimondo et al., 2006).

Isolated hearts

Male Wistar rats (n= 8; body-weight 450-550 g) were heparinized (2.500 U. i.m.) and 10 min after anesthetized with urethane (1 g/kg i.p.). Then, the hearts were rapidly excised and attached to the perfusion apparatus and retrogradely perfused with oxygenated Krebs-Henseleit buffer supplemented with 5 μg /mL lidocaine and gassed with 95% O_2 and 5% CO_2 (28; 25). The hearts were instrumented as previously described (Pagliaro et al., 2003). A constant flow was adjusted with a proper pump to obtain a typical coronary perfusion pressure of 80-85 mm Hg. Also temperature (37° C) of perfusate and hearts was strictly controlled and kept constant throughout the experiment.
After a period of stabilization (20 min), the left descending coronary artery (LDCA) was occluded for 30 min followed by 10 min of reperfusion before the MSC-GFP were injected.

Hearts were removed from the apparatus for histological fixation at four different various times after MSC-GFP injection (5 min, 2 hours, 4 hours, 6 hours after injection: n= 2 for each time point).

MSCs transplantation

The MSCs at P6 were washed with phosphate-buffered saline (PBS) and detached by incubation with 0.25% trypsin and 0.1% EDTA (Sigma, St Louis, MO, USA) for 5 to 10 minutes at 37°C. Complete medium was added to inactivate the trypsin. The cells were centrifuged at 800g for 5 minutes, counted using a burker's chamber and $1x10^6$ cells were resuspended in 300 µl of PBS. These cells were then injected in the wall of the left ventricle close to the border zone of the previously ischemic area. In this model the ischemic zone is easily recognizable as it turn pale-colored in few seconds after coronary occlusion.

Light and confocal imaging

Tissues were fixed with 4% Paraformaldehyd for 2h and then washed in a solution of 0.2% glycin in 0.1 M phosphate buffer (pH 7.2) for 30 min. Then the tissues were embedded in increasing solutions of sucrose (7.5% for 1h - 15%for 1h - 30% O/N) in 0.1 M phosphate buffer and then in a solution of PBS/OCT 50% for 30 min. Finally, the tissues were embedded in 100% OCT and stored at -80°C. The slides were observed with a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany), which incorporates two lasers (Argon and HeNe) and is equipped with an inverted Axiovert 100M microscope.

After confocal observation and digital image acquisition, the slides with evidence of GFP-positive cells were stained with haematoxylin and eosin and observed and photographed with a DM4000B microscope equipped with a DFC320 digital camera and a IM50 image manager system (Leica Microsystems, Wetzlar, Germany)a Leica DFC 320 light microscope.

Finally the images were superimposed to allow an accurate morphological description of GFP-positive cells.

In addition, to verify that the observed fluorescence was not due to the tissue autofluorescence of the heart, some slides were immunostained with a-GFP antibody (polyclonal, rabbit, 1:100, Abcam, Cambridge, UK) followed by incubation with anti-rabbit biotinylated secondary antibody (Dako, Milan, Italy). Sections were then processed with Vectastain ABC kit (Vector, Burlingame, CA, USA) with peroxidase, revealed with diaminobenzidine (Sigma, St Louis, MO, USA), and observed and photographed with the DM4000B light microscope.

Drugs and chemicals

The drugs used in these experiments were all freshly dissolved at the required concentration in the perfusion buffers. Most of the compounds were obtained from Sigma Chemical (St Louis, MO, USA), heparin from Roche (Milan, Italy).

Results

The strong green autofluorescence makes transplanted MSCs easily recognizable inside the injection cavity in the myocardium where they are all concentrated.

Haematoxylin and eosin staining on the same slide reveals the clear morphological difference between these cells and the surrounding myocardiocytes.

Observations made on infarcted hearts removed 2, 4 and 6 hours after MSC transplantation revealed that most MSCs remained concentrated in clusters located near the site of injection.

Sequential direct GFP confocal imaging followed by immunostaining with anti-GFP antibody shows that the observed fluorescence was not due to autofluorescence of the myocardiocytes.

Discussion

Results of the present study showed that the isolated perfused heart model can be successfully used to investigate primarily the early behaviour of myocardial transplanted cells inside the myocardium/cellular mechanisms of homing, excluding the presence of potentially confounding external factors, including such as endothelial/neutrophil interaction and neuro-hormonal control. This model can also be used to study the role of each external factor by administering them to the isolated heart individually or in association.

This is the first time that isolated beating hearts were used to study the early aspects of homing of GFP-stable transfected MSCs injected in the left ventricular wall. Since the initial phase after injection is critical for cell survival (Rubart M and, Field, LJ. 2005) adequate knowledge of the early homing of MSCs in the infarcted myocardium may improve the outcome of SC therapy which has been known to produce negative and positive results (Orlic et al., 2001; Smits et al., 2005). Therefore, to study the initial homing features of injected MSCs we have implemented a simple method that can easily be reproduced in other laboratories with experience in *ex vivo* heart preparation.

While our main goal was to implement this new model, the results that we obtained showed that in infarcted hearts MSCs, which maintain their typical round shape, concentrate in the ischemic border zone. These findings in agreement with the findings of other authors, which have shown that chemo-attractant factors are released by the infarcted area (Sauer et al., 2001).

The finding that, in infarcted hearts, MSCs concentrate in the border zone of ischemia, where they could proliferate as undifferentiated (round-shaped) cells, supports the idea that they may regenerate infarcted myocardium (Orlic et al., 2001).

As regards methodological issue, the easiness of imaging of GFP transplanted cells is also a very important point in favour of the proposed experimental model. Actually, *in vivo* imaging of GFP is particularly difficult in the heart because of autofluorescence of this tissue. On the contrary, in the isolated heart true GFP fluorescence can be easily distinguished from the surrounding autofluorescence.
Isolated heart preparation enables the effective, reproducible, and economic study over a wide range of experimental conditions. For example, the isolated heart can be readily subjected to conditions of global no-flow ischemia for studying of resistance to ischemia. This situation avoids the potential contribution of collateral flow supplied from nonischemic capillary beds in conditions of regional ischemia that may influence resistance to ischemia. In addition, temperature is strictly controlled and constant ventricular volume eliminates differences in loading conditions.
Isolated beating hearts are inadequate for a long period of observation since they cannot be sustained for reperfusion periods longer than few hours (Pagliaro et al., 2003; Dawn et al., 2005). However, it must be considered that in this model coronary flow is 10-12-fold higher than in the *in situ* preparation (Pagliaro et al., 2003; Bullard et al., 2005). This higher flow causes the intercellular milieu to change much more rapidly so that the stem cells found after 4-6 hours in the isolated heart have been exposed to a number of milieu variations that in *in situ* conditions may occur after days from the event (Yang et al., 2005). Thus it may be argued that in a few hours an isolated heart model can give information that *in vivo* may require a longer period of time, because rapid changes in the milieu are likely to induce faster response of MSCs.
The isolated heart model is thus particularly interesting for studies that consider myocardial infarction as target of MSC therapy. In fact, important determinants of infarct size are under the control of the researcher, whereas *in vivo* they are extremely variable. Neuro-humoral influences, as well as many other potentially influencing factors, could be tested to ascertain their role.
Taken together, our results suggest that isolated beating heart model can be a simple and effective method to study early stem cell homing in a cardiac environment in which the various intrinsic and extrinsic factors may be strictly controlled.

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