Cardiac precursors in human bone marrow and cord blood: *in vitro* cell cardiogenesis

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Background. **Cell transplantation has come of age but numerous questions still remain. Which type of cell should be used? Cardiac precursors are present in mouse bone marrow and used to repair the infarcted myocardium in mice. We searched for these precursors in human bone marrow and analyzed gene expression patterns in cells induced to differentiate *in vitro*.**

Methods. **Cells from human bone marrow were isolated and cultured in medium supplemented with autologous serum and 5% CO_2. Cell characterization was performed by immunocytochemical analysis. mRNA was isolated and retrotranscribed. The active genes were detected with polymerase chain reaction by using specific oligonucleotides.**

Results. **Some inducers pushed the cell through different stages of cardiogenesis, with expression of cardiac transcriptional activators and structural proteins. Some combinations of stimuli were able to drive cells to advanced stages of cardiogenesis.**

Conclusions. **These studies lead to an exact description of *in vitro* cardiogenesis in humans. Our aim was also to assess the residual proliferative capacity of cells and to enhance the differentiation efficiency, thus maximizing their repair capacity and the likelihood that they functionally integrate with the surrounding cardiac tissue.**

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Background

The cardiac tissue is apparently unable to regenerate and repair cell damage after infarction. However, recent studies have shown that mobilization of bone marrow (BM) cells by cytokine cocktails results in homing of precursors into the damaged tissue[1] and that cardiac-specific stem cells resident in the adult heart may be involved in slow heart tissue renewal[2]. The extensive damage resulting from infarction is beyond the possibilities of this putative homeostatic mechanism, which is most probably active in low-rate cardiac tissue maintenance. However, the existence of cardiac-specific precursors in human BM and possibly in umbilical cord blood (UCB) paves the way to a possible cell therapy approach for the restoration of cardiac function after infarction. The BM is in fact a potentially convenient source of autologous stem cells. Preliminary studies in animals have addressed the issue of the ability of these cells to home in the site of infarction and to engraft into the damaged tissue. There is however no clear indication of whether true "hematopoietic" (i.e. $CD34^+$) stem cells or $CD34^-$ cells are particularly enriched in cardiac cell precursors. In addition, the issue of adult stem cell plasticity versus precursor heterogeneity is still a matter of strong debate[3-5]. Data supporting either the $CD34^+$ or $CD34^-$ origin of cardiac precursors may be found in the recent literature, with apparently comparable experimental evidence[6,7].

Cardiac cell differentiation, as other differentiation pathways, depends on a wide array of soluble growth and differentiation factors, on the activation of cardiac-specific genes, on interactions of differentially expressed integrins with extracellular matrix components[7], on intercellular contacts and anatomical as well as functional integration. Most of these features have not been clearly defined in humans, or even in rodents: therefore, the study of cardiac stem cell precursors from human UCB and BM may lead to both an improved knowledge on the biology of human cardiac cell differentiation, as well as in practical outcomes, including the possibility of a safer autologous or histocompatibility-matched stem cell transplantation.

Studies in animal models have shown that cell transplantation may lead to an improved cardiac function[8]. The cell transplantation approaches performed so far have made use of either autologous skeletal satellite cells isolated from muscle biopsies, or of autologous BM as a source of cardiogenic stem cells. Current literature suggests that the potential benefit of post-infarct cell transplantation using skeletal muscle cells might be due to a greater left ventricular remodeling rather than to an increase in synchronous contractility. However, these data might need to be confirmed in the long-term follow-up: in fact, skeletal muscle cells differ from cardiac cells not only in terms of their morphological and physiological features, but also in the expression of different repertoires of functionally relevant gap junction components, namely those coded for by connexin genes. Therefore, one might expect that extensive cell replacement of cardiac tissue with skeletal muscle cells would lead to a relatively low degree of integration of the transplanted tissue within the recipient organ, thus resulting in an asynchronous function and arrhythmias. The putative ideal source for cell transplantation would therefore be a cell able to home naturally into the infarct region, that establishes functional physiological connections with the recipient tissue, and integrates anatomically and electrically within the heart, supporting the overall synchronous contractility of the restored organ. So far, there is little evidence about the cell types and the procedures that are best suitable in terms of homing, successful engraftment and efficient mechanical contribution to cardiac function after cell transplantation.

Our aim was to address the problem of the *in vitro* differentiation of human UCB and BM stem cells into cardiomyocytes. UCB is potentially a very convenient source of human hematopoietic stem cells: we show in this paper that UCB cells with mesenchymal properties and cardiac potential may be recovered from this source with yields comparable to those obtained from the BM. Due to the fact that this source is easily available, and that it is closer to the embryo than adult sources, we decided to use UCB cells as a model system for the assessment of the experimental conditions for cell differentiation and cultivation. It is clear that this source is currently not suitable for transplantation, due to the need for histocompatibility matching between the donor and the recipient. However, the conditions that have been set up in this system could be transferred to the autologous BM setting for potential clinical use. We addressed the issue of precursor plasticity vs heterogeneity by establishing clonal cell cultures of precursor cells and by checking gene expression differences at the clonal level, upon induction of cell differentiation.

Methods

Umbilical cord blood and bone marrow cells: selection of a CD34$^-$ fraction. Ten ml of UCB or BM were carefully layered over a 5 ml Ficoll-Paque solution and centrifuged at 800 × g for 20 min. The mononuclear cell ring at the plasma/Ficoll interface was recovered and the red blood cell fraction containing granulocytes was discarded. The mononuclear cells were exposed to a cocktail of antibodies for rosette depletion of the lineage-positive cells (Stem Cell Technologies, Vancouver, Canada). The sample was divided into two aliquots, one of which was rosetted with antibodies against CD34 to enrich for CD34$^-$ precursors, while the other was left untreated.

Cell cultures. The cells were suspended in RPMI 1640 tissue culture medium (Euroclone, Pero, Italy) supplemented with L-glutamine and 10% fetal calf serum (Euroclone) or in autologous serum when applicable. Mesenchymal cell medium (Cambrex, Bergamo, Italy) was also used in these preliminary experiments and found to provide the best results in terms of clonal growth and yield of cells positive for cardiac-specific gene expression. Cells were cultured both under clonal conditions (limiting dilution plating in microtiter plates) and in bulk cell cultures at 10^5 cells/ml. All the cultures were kept in a CO_2 incubator (5% CO_2, 37°C, 95% humidity).

Differentiation into cardiac cells. We made use of the extracellular matrix proteins fibronectin and laminin to check whether they could influence the fate of the putative cardiac progenitors present in our clonal cell cultures. Either fibronectin, laminin (Sigma, Milan, Italy), or the corresponding synthetic peptides (Sigma) responsible for the binding to differentially expressed integrins was used at 10 and 1 μg/ml respectively, for a 7-day culture period. Total RNA was extracted from the different clones and analyzed by means of reverse transcriptase-polymerase chain reaction (PCR) as described below.

RNA extraction, reverse transcription and polymerase chain reaction. The RNA of $10^5 – 4 \times 10^6$ cells was totally extracted using a commercially available kit (RNAqueous, Ambion, Milan, Italy). Reverse transcription was performed in standard conditions using the Promega (Florence, Italy) RT kit and following the manufacturer's instructions. PCR was performed using Promega Taq Polymerase and primers for specific genes obtained from Invitrogen. We decided to assess the expression of different genes related to cardiac differentiation, including terminal markers such as Titin and the cardiac myosin heavy and light chains, transcriptional activators such as Nkx 2.5, GATA-4, E-HAND, D-HAND, and MEF-2A (see Appendix). The primers were chosen using the NCBI database as a source of the sequences and the Geneworks program (Intelligenetics) for primer selection using default parameters and restricted to primers belonging to different exons in order to avoid amplification due to possible genomic DNA contamination. The specificity and uniqueness of the different primers for the desired gene were assessed using BLAST.

Electrophoresis. Amplified DNA was run at 100 V for 30 min in a submarine electrophoretic cell in 1% agarose gels and dissolved in TBE (Tris buffer-EDTA) containing 20 ng/ml ethidium bromide gel for DNA visualization. The gels were UV-transilluminated and photographed with Polaroid equipment.

Results

Clonal cell cultures obtained from the bone marrow and umbilical cord blood. Several clones attaching to cell culture substrates were obtained both from BM and UCB cultures after 15-20 days of cultivation under non-induced conditions (Fig. 1). The cloning efficiency was low (1/2000 cells), but once established, the cells grew firmly attached to the substrate and rapidly reached semi-confluence. No difference was found in the clonal yield using $CD34^+$-depleted cell fractions, indicating that the clones were of $CD34^-$ origin. Clones were replicated into three subcultures in a 24-well plate (Corning, Milan, Italy) for further differential treatment and RNA extraction. The morphology of the cells was consistent with the mesenchymal precursors described by Verfaillie[7]. The cells did not stain with FITC-labeled anti-CD34 monoclonal antibodies.

Different clones heterogeneously express cardiac-specific markers. Examples of PCR tests performed in induced vs non-induced clones for a series of cardiac-specific markers are shown in figures 2, 3 and 4: it was evident that the ability of different clones to undergo cardiac-specific differentiation, as assessed through analysis of gene expression, was heterogeneous. As shown in table I, only one out of 9 different clones tested was able to express the myosin heavy chain mRNA after induction, but failed to express Titin. However, they were all shown to express mRNAs coding for transcriptional activators to different degrees. Whether this heterogeneity reflects the short period of exposure to inducers of cell differentiation is currently being assessed by longer-term cultivation. Further studies are also being performed using different inducers and combinations of inducers, added simultaneously or in successive rounds, in order to clarify to what extent the *in vitro* cardiac differentiation process may be driven to completion.

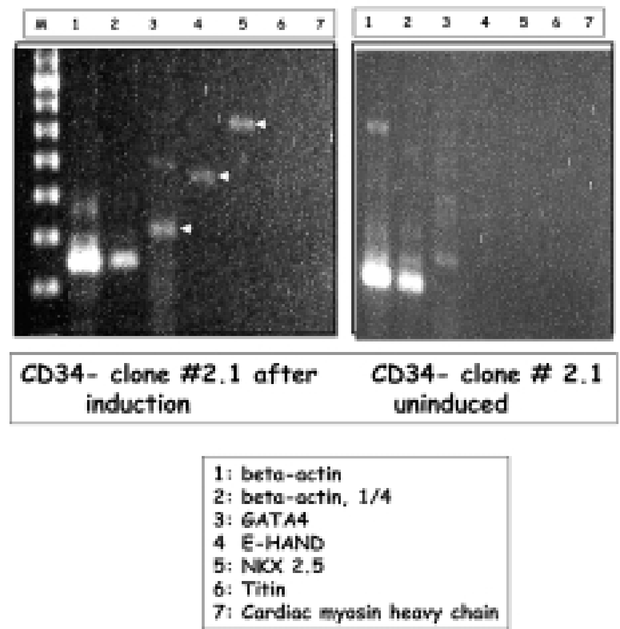

Figure 2. *Polymerase chain reaction analysis of specific transcripts from the mesenchymal cell clone 2.1 obtained from a umbilical cord blood culture. For the experimental conditions, see the Methods section.*

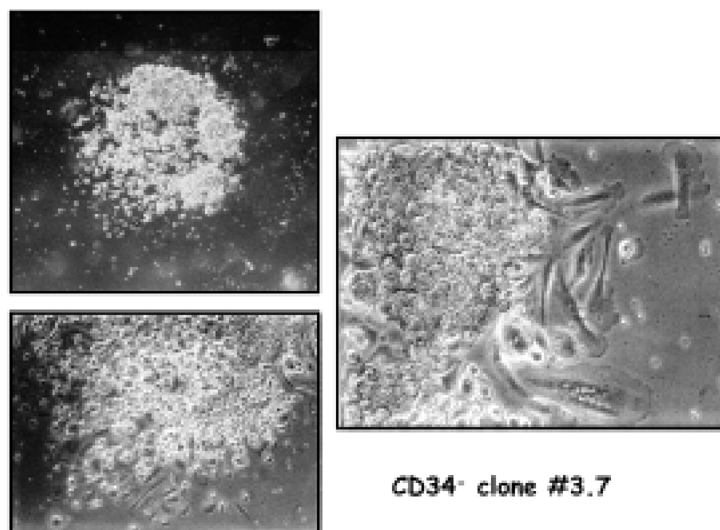

Figure 1. *Phase contrast microscopy of a representative clone of mesenchymal cells from human bone marrow at different magnifications: counterclockwise, 100×, 200×, 400×.*

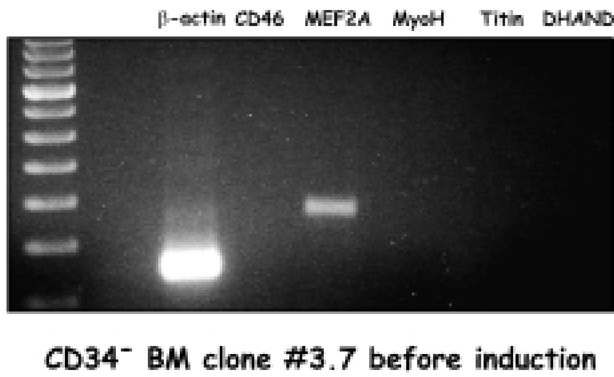

Figure 3. *Polymerase chain reaction analysis of specific transcripts from the mesenchymal cell clone 3.7 obtained from an non-induced bone marrow (BM) culture. For the experimental conditions, see the Methods section.*

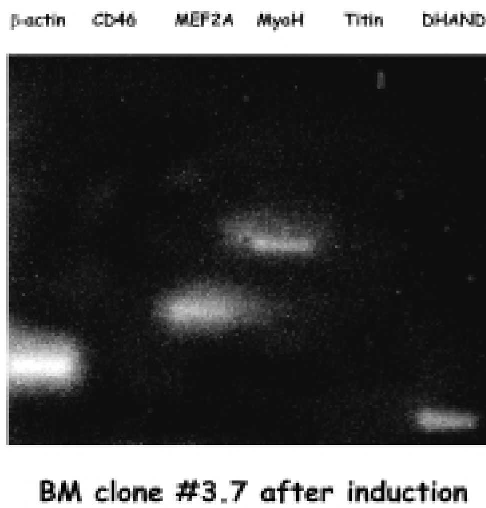

Figure 4. *Polymerase chain reaction analysis of specific transcripts from the mesenchymal cell clone 3.7 obtained from a bone marrow (BM) culture and induced with fibronectin. For the experimental conditions, see the Methods section.*

Discussion

Stem cell research is currently addressing the issue of finding a suitable source for cell-based transplantation therapy. Due to the ethical restrictions in the use of embryonic stem cells, several attempts are being followed in which adult tissues are used as the source of pluripotent cell precursors for tissue substitution therapies. Autologous sources, such as the BM and satellite cells from skeletal muscle are promising sources of cells to be used in healing cardiac tissue after infarction. This approach has the clear advantage of not being burdened by possible complications arising from HLA mismatching: the cells may in fact be drawn from the patient's BM or skeletal muscle, cultured and amplified, brought to a cell density compatible with the extent of tissue damage and characterized *in vitro* before treatment, as far as their differentiation potential is concerned. The drawback is the need of setting up "personal" cell populations, which may only be used for the donor. Several factors may limit the applicability of this approach to clinical use: first of all, the use of cells belonging to a different lineage, such as the skeletal muscle, might not represent the ideal solution as far as the perfect anatomical and functional integration within the recipient heart is concerned: differences in electrical excitability, as well as in connective properties between cardiac resident cells and skeletal transplanted cells might reduce their functional integration and lead to a patch of tissue with autonomous electrical properties as compared to the rest of the organ. On the other hand, the conditions for proper cultivation of cardiac-committed cells from human BM precursors are poorly described in the current literature. This is especially true if compared to the rodent system. There is no apparent concordance between different laboratories on issues such as the surface antigen composition of cardiac precursors, the plasticity or heterogeneity of committed cells and the stimuli that lead to cell differentiation *in vitro*.

Cell sources which are not matched with the patients' tissues at the HLA level may be envisioned for future clinical use: in particular, embryonic stem cells and cells taken from the UCB. Legal restrictions in the use of embryos for therapeutic purposes make the use of embryonic stem cells highly unlikely in the near future. UCB does not imply such limitations and could be used for this purpose, provided that sufficient amounts of cells can be obtained and cultured for long periods, so as to establish a large cell repository, which can be used for different patients. In this case, the use of these

Table I. Summary of the gene expression profiles of different clones obtained from the umbilical cord blood and bone marrow.

Clone	GATA-4	MEF-2A	Nkx 2.5	E-HAND	D-HAND	MyoH
3.1	+	–	–	–	–	–
3.2	+	–	–	–	–	–
3.3	+	+	+	–	–	–
3.4	+	–	–	–	–	–
3.5	+	–	–	–	–	–
3.6	+	–	–	–	–	–
3.7	+	+	+	+	+	+
2.1	+	+	+	+	–	–
2.2	+	–	–	–	–	–

cells for human therapy implies that the patients should be immunologically suppressed for the rest of their lifetime. It is possible that immunosuppressive therapies will be greatly improved in the future, thus facilitating the successful engraftment of non-perfectly matched tissues.

The data reported in this article show that the UCB may be proposed as a suitable source of cardiac-committed precursors: no significant differences were found in the yields of mesenchymal cell clones using UCB or BM as the source of cells. Moreover, we have found that the behavior of different clones in response to the induction of cell differentiation by fibronectin and laminin is apparently heterogeneous. Only a small proportion of the clones obtained appear to express cardiac-specific markers in the experimental setting applied. This might be due to the use of a limited amount of differentiation factors, but is anyway indicative of precursor heterogeneity. Further studies are therefore on the way in our laboratory to enhance the efficiency of cell differentiation. Taken together, these data suggest that plasticity at the precursor cell level might be a rare event, and that clonal heterogeneity of the adult precursor cell pool is already established in cells obtained from the BM and the UCB. We are currently investigating the expression of cardiac connexins both at the mRNA and at the protein levels in these cultured clones, as well as the electrical excitability of the cultured cell clones after induction of cell differentiation. This is an important feature that must be determined in order to ensure that the *in vitro* differentiated cells will establish successful functional connections with the resident tissue.

We suggest therefore that attempts to transplant whole, unsorted populations of BM and UCB cells take into account that only a small proportion of the transplanted cells will home to the desired organ and undergo cardiac-specific cell differentiation. Most cells will probably home to different organs or return to the BM, without colonizing the damaged heart. Further studies are required to assess the release of factors from the damaged tissues, which might attract stem and progenitor cells to the site of the lesion.

Appendix

Polymerase chain reaction primers used

1. Cardiac myosin light chain, forward primer
   Sequence (5' to 3'): TTC CAA GGA GGA GGT TCA CC
2. Cardiac myosin light chain, reverse primer
   Sequence (5' to 3'): TTC CAA CTG TAG GAT GTG CG
3. Titin, forward primer
   Sequence (5' to 3'): AGA CAT GGT CGA CCG TTA CC
4. Titin, reverse primer
   Sequence (5' to 3'): TAC TCT GTC CAG CGA TCT GC
5. Cardiac myosin heavy chain, forward primer
   Sequence (5' to 3'): CAC AAG TGC CTC TAA CGT GG
6. Cardiac myosin heavy chain, reverse primer
   Sequence (5' to 3'): GAG AGT GGC TTC AAC TTC GG
7. Nkx 2.5 forward primer
   Sequence (5' to 3'): TTC AAG CAA CAG CGG TAC C
8. Nkx 2.5 reverse primer
   Sequence (5' to 3'): TAA CCG TAG GGA TTG AGG CC
9. E-HAND forward primer
   Sequence (5' to 3'): TCA AGG CTG AAC TCA AGA AGG
10. E-HAND reverse primer
    Sequence (5' to 3'): AAT TAG AGA AGA CGG CGT CG
11. GATA-4 forward primer
    Sequence (5' to 3'): CTT GGA ACA GTC TGG TCT TGG
12. GATA-4 reverse primer
    Sequence (5' to 3'): ACA GGA GAG ATG CAG TGT GC
13. MEF-2A forward primer
    Sequence (5' to 3'): TTG AGG CTC TGA ACA AGA AGG
14. MEF-2A reverse primer
    Sequence (5' to 3'): GCA TTG CCA GTA CTT GGT GG
15. D-HAND forward primer
    Sequence (5' to 3'): GAC TCA GAG CAT CAA CAG CG
16. D-HAND reverse primer
    Sequence (5' to 3'): TTG ATC TCT GCC TTG AAG GC
17. Beta-actin forward primer
    Sequence (5' to 3'): CAC GAT GGA GGG GCC GGA CTC ATC
18. Beta-actin reverse primer
    Sequence (5' to 3'): TAA AGA CCT CTA TGC CAA CAC AGT

References

1. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001; 410: 701-5.
2. Anversa P, Leri A, Kajstura J, Nadal-Ginard B. Myocyte growth and cardiac repair. J Mol Cell Cardiol 2002; 34: 91-105.
3. Dorschkind K. Multilineage development from bone marrow stem cells. Nat Immunol 2002; 3: 311-3.
4. Wulf GG, Jackson KA, Goodell MA. Somatic stem cell plasticity: current evidence and emerging concepts. Exp Hematol 2001; 29: 1361-70.
5. Tosh D, Slack JM. How cells change their phenotype. Nat Rev Mol Cell Biol 2002; 3: 187-94.
6. Fukuda K. Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. Artif Organs 2001; 25: 187-93.
7. Verfaillie CM. Hematopoietic stem cells for transplantation. Nat Immunol 2002; 3: 314-7.
8. Hagege AA, Carrion C, Menasche P, et al. Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy. Lancet 2003; 361: 491-2.