Scaffold construction for effective transfer of cardiac stem cells to the damaged heart

Konstrukcja rusztowań dla efektywnego transferu komórek macierzystych serca do uszkodzonego mięśnia sercowego

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Abstract

Specific cardiac stem/progenitor cells (CSCs/CPCs) are multipotent and clonogenic, giving rise to cardiomyocytes, smooth muscle cells and endothelial cells. These cells can be used in cell therapy for the treatment of diseased heart tissue, but there is a need for an effective targeting system that directs the cells to the infarcted area. The aim of this study was to monitor changes in CSC/CPC morphology and the percentage of c-kit- and MDR-1-positive cells after culturing on various substrata and in different media.

Myocardial tissue samples were taken from explanted hearts under sterile conditions. After the cells had grown to confluence, they were detached by gentle enzymatic digestion and seeded on poly-D-lysine-coated dishes. After a period of one week, the cells were transferred onto different substrata and cultured with growth factors and without growth factors.

Cells transferred to the culture dishes coated with different substrata presented differences in morphology and in the percentage of c-kit- and MDR-1-positive cells. Higher percentages of c-kit- and MDR-1-positive cells were observed on the fibronectin-coated dishes cultured without growth factors. The data obtained should be useful for the construction of a scaffold dedicated to the effective transfer of c-kit- and MDR-1-positive cells to the damaged heart.

Key words: cardiac stem cells, scaffold, heart disease, cell therapy.

Streszczenie

Sercowe komórki macierzyste i progenitorowe (ang. *cardiac stem/progenitor cells* – CSC/CPCs) są grupą komórek multipotencjalnych, zdolnych do wielokrotnej replikacji i różnicowania się w kardiomiocyty, komórki endothelium oraz komórki mięśni gładkich. Z tego względu wydaje się, że komórki te mogą w przyszłości znaleźć zastosowanie w leczeniu chorobowo uszkodzonego mięśnia sercowego. O efektywności leczenia decydować mogą takie czynniki, jak efektywna izolacja CSC/CPCs i ich namnażanie w warunkach *in vitro*. Niemniej jednak istotne jest opracowanie optymalnej metody transferu tych komórek do uszkodzonego mięśnia sercowego.

Celem badań była ocena zdolności wzrostu izolowanych komórek macierzystych i progenitorowych serca na zróżnicowanych podłożach, które mogą mieć potencjalne zastosowanie do tworzenia bezkomórkowych rusztowań do nahodowywania i transferu CSC/CPCs do uszkodzonego mięśnia sercowego.

Do badań wykorzystywane były fragmenty eksplantowanego mięśnia sercowego. Izolowaną tkankę mięśnia sercowego cięto na fragmenty o wielkości 1–2 mm^3, poddawano trawieniu enzymatycznemu z użyciem roztworów 0,2% Trypsyna/EDTA i 0,1% kolagenoza IV. Następnie trawione fragmenty tkanki płukano w medium IMDM, przenoszono do naczyń hodowlanych i hodowano w standardowych warunkach. Po okresie ok. 2–3 tygodni z przyczepionych fragmentów tkanek uzyskiwano monowarstwę komórek, wśród których obserwowano małe okrągłe komórki, jasne w kontraście fazowym. Komórki następnie przenoszono na płytki hodowlane opłaszczone poli-D-lizyną i hodowano w medium suplementowanym czynnikami wzrostu. Wydaje się, że o właściwym wzroście, migracji i różnicowaniu komórek macierzystych serca decydować mogą m.in. oddziaływania z elementami macierzy zewnątrzkomórkowej, co może mieć istotne znaczenie

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w konstruowaniu nośników dla efektywnego transferu komórek macierzystych w obszar uszkodzonego mięśnia sercowego.

Słowa kluczowe: komórki macierzyste serca, rusztowania komórkowe, choroby serca, terapie komórkowe.

Introduction

In recent years, much work has focused on elucidating the biology of stem cells and their mechanisms of action with a view towards using them to regenerate damaged organs, including the treatment of myocardial infarction. For this reason, great interest and expectations are associated with the discovery of cardiac stem cells (CSCs), a group of newly discovered multipotent cells that are capable of multiple rounds of replication and differentiation into cardiomyocytes, endothelial cells and smooth muscle cells. These cells are characterized by the presence of the surface proteins c-kit (CD117), MDR-1 and Sca-1. The discovery of CSCs revolutionized the perception of the heart muscle as an organ incapable of regeneration. Currently, it is believed that aging heart muscle cells, including cardiomyocytes, coronary vessel-forming cells and stromal cells, are constantly replaced by new generations of corresponding cells that are fully functional. However, the great potential for cardiac regeneration appears to be insufficient with respect to ischemia. It is possible that the microenvironment of the infarcted heart inhibits the recruitment of residual stem cells. Because the treatment of pathologically damaged heart muscle by cell therapy involves repopulating the infarcted region with a sufficient number of CSCs, these cells can be isolated from heart muscle and expanded *in vitro*. However, the effectiveness of this type of therapy is largely determined by the efficiency with which these cells localize to the damaged heart muscle. The use of the scaffolds is one method of administering stem cells to the damaged heart. Note that the microenvironment of the cells, direct signal transduction by matrix molecules through integrin receptors and physical properties, such as scaffold stiffness, can influence the fate of stem cells and their differentiation process. The aim of this study was to evaluate the impact of different types of substrata used in the preparation of the scaffold on the growth ability and surface receptor expression of CSCs. Our results should contribute to the development of an appropriate scaffold dedicated to effective CSC transfer to damaged heart muscle.

Material and methods

Cardiosphere-forming cell isolation and culture

Cardiac stem cells were isolated using the enzymatic method according to Messina and colleagues. Briefly, cardiac muscle tissue derived from explanted heart was cut into 1-2 mm^3 pieces and washed in phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} several times to remove the contaminating fat and red blood cells. After washing, the tissue was digested using a solution of 0.2% trypsin/ EDTA plus 0.1% collagenase IV for 5 min at 37°C; the procedure was repeated 3 times. After isolation, the cells were discarded, and the remaining tissue fragments were washed in IMDM medium (Iscove's Modified Dulbecco's Medium) supplemented with 10% fetal calf serum (FCS), 20 U/ml penicillin G, 20 mg/ml streptomycin and 2 mmol/L L-glutamine. After washing, the tissue fragments were seeded onto culture dishes and incubated at 37°C with 5% CO_2. After a period of approximately 2-3 weeks, a fibroblast-like layer was generated, over which small, round, phase-bright cells were observed. The phase-bright cells were collected through two washes in PBS without Ca^{2+} or Mg^{2+} (1-2 min), one wash in 0.53 mmol/l EDTA, and finally one wash in a 0.5 g/l trypsin and 0.53 mmol/l EDTA solution (2-3 min) at room temperature. The harvested cells were transferred to culture plates coated with poly-D-lysine and cultured in a mix of IMDM medium (35%) and Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium (65%) supplemented with 1% L-glutamine, 20 ng/ml bFGF, 2% B27, 4 ng/ml cardiotrophin, 10 ng/ml EGF, 40 nM/l thrombin, and 0.2% mercaptoethanol.

Secondary culture of cardiosphere-forming cells on different substrata

After one week, cells that failed to adhere to the poly-D-lysine coated dishes were collected by centrifugation and treated with StemPro Accutase Cell Dissociation Reagent in accordance with the manufacturer's protocol to dissociate the cardiospheres into single cells. Afterwards, the cells were plated onto dishes coated with fibronectin, collagen IV, gelatin and laminin and cultured with a) IMDM medium (35%) and DMEM/Ham's F12 medium (65%) supplemented with 1% L-glutamine without growth factors or b) IMDM medium (35%) and DMEM-Ham's F12 medium (65%) supplemented with 1% L-glutamine, 20 ng/ml bFGF, 2% B27, 4 ng/ml cardiotrophin, 10 ng/ml EGF, 40 nM/l thrombin, and 0.2% mercaptoethanol.
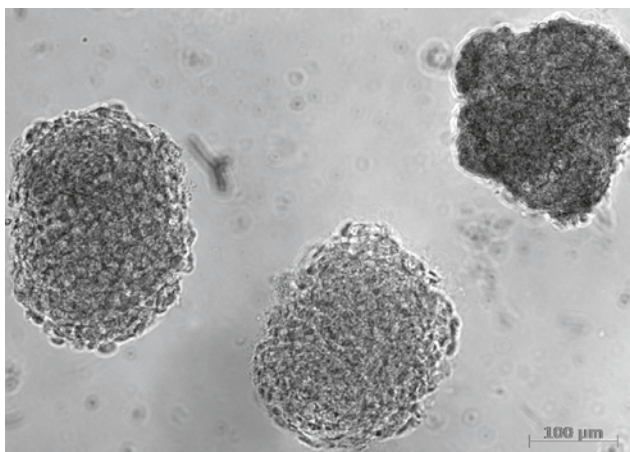
Flow cytometry

Cardiosphere-forming cells from secondary cultures on poly-D-lysine and cells cultured on fibronectin-, collagen IV-, gelatin- and laminin-coated dishes and cultured with a) IMDM medium (35%) and DMEM/Ham's F12 medium (65%) supplemented with 1% L-glutamine without growth factors or b) IMDM medium (35%) and DMEM-Ham's F12 medium (65%) supplemented with 1% L-glutamine, 20 ng/ml bFGF, 2% B27, 4 ng/ml cardiotrophin, 10 ng/ml EGF, 40 nM/l thrombin, and 0.2% mercaptoethanol were prepared for flow cytometry using a Beckman Coulter FC 500 flow cytometer with

MXP software (Beckman Coulter). The following monoclonal antibodies were used: c-kit, Nkx 2.5, MDR-1, GATA-4, CD90, CD45, CD31, CD73, CD105, CD144, Lin-1, Lin-2, and Lin-3.

Analysis of cell morphology – fractal analysis

Phase contrast microscopy was used to directly follow cell morphology in cultures over a period of 8 days. Images were taken 24 h after seeding the cells on fibronectin-, collagen IV-, gelatin- and laminin-coated dishes and on the 8th day of the experiment. An inverted AxioObserver microscope was used for image acquisition, and AxioVision 1.4 software was used for image processing. Additionally, binary (black and white) image processing was performed using a public domain NIH image program (ImageJ) developed at the US National Institute of Health. To quantify the complex morphology of the cells in culture, fractal dimension (FD) analysis via the box-counting method was performed using Fractalyse 2.4.1 software.

Statistical analyses

The results were reported as mean values ± SD. The basic statistics employed the STATISTICA 7.0 package. The data were tested for homogeneity of variance using Levene's test of equality of error variances. Normality was checked by the Kolmogorov-Smirnov test. Whenever a significant effect was observed, post hoc comparison was performed with the Tukey test to check differences between experimental groups.

Results

### Primary and secondary cell culture

Explanted human hearts were stored in high-potassium cardioplegic solution and processed within 1 h. The tissue was cut into 1-2 mm^3 pieces, and the isolation procedure was performed. After a period of approximately 2-3 weeks in the primary culture, a layer of fibroblast-like cells was observed over which small, round, phase-bright cells were observed. The phase-bright cells were collected by mild digestion and transferred to poly-D-lysine-coated dishes. After seeding on poly-D-lysine-coated dishes, the cells formed a cardiosphere (Fig. 1).

Fig. 1. The cells in secondary culture on poly-D-lysine coated dishes form a cardiosphere structure
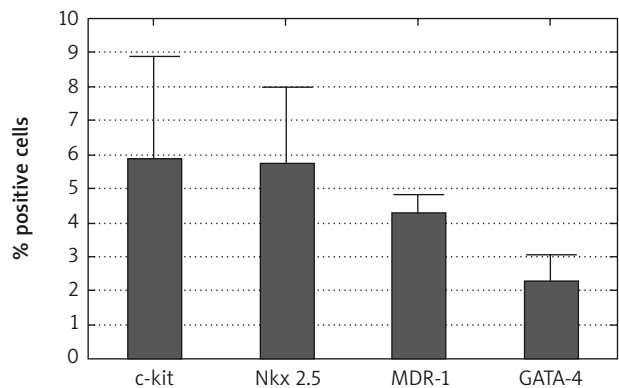
Fig. 2. Flow cytometry analysis of human CSCs/CPCs in secondary culture on poly-D-lysine coated dishes – percentage of c-kit, Nkx-2.5, MDR-1, GATA-4 positive cells
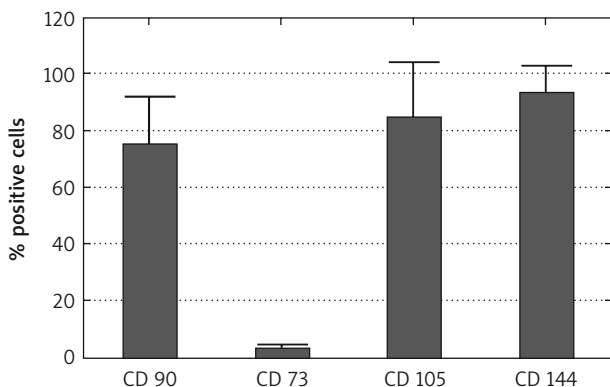
Fig. 3. Flow cytometry analysis of human CSCs/CPCs in secondary culture on poly-D-lysine coated dishes – percentage of CD90, CD73, CD105, CD144 positive cells
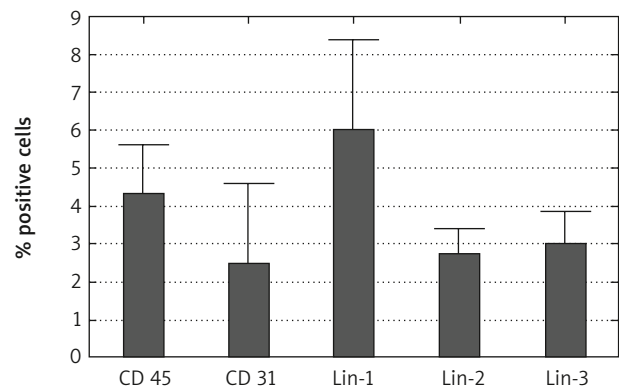
Fig. 4. Flow cytometry analysis of human CSCs/CPCs in secondary culture on poly-D-lysine coated dishes – percentage of CD45, CD31, Lin-1, Lin-2, Lin-3 positive cells

The cardiospheres in secondary culture on poly-D-lysine-coated dishes were loosely adherent. The cardiosphere-forming cells were c-kit-positive in the core and CD105-positive in the periphery.

Flow cytometric analysis of the cells in the secondary culture indicated that nearly 7% of the cells were c-kit positive and that approximately 6% were Nkx-2.5 positive. Expression of the MDR-1 and GATA-4 receptors was observed in the secondary culture (Fig. 2). Approximately 80% of the cardiosphere-forming cells were CD90-positive, and these cells were also largely (approximately 90%) CD105- and CD144-positive. The CD90-, CD105- and CD144-positive cells presented a mesenchymal or fibroblast phenotype (Fig. 3). Only a low percentage of the cardiosphere-forming cells were positive for the hematopoietic lineage markers CD45, CD31, Lin-1, Lin-2, and Lin-3 (Fig. 4).

Cardiosphere-forming cells after transfer to different substrata: fibronectin, collagen IV, laminin, gelatin

Cells derived from the secondary culture on poly-D-lysine-coated dishes were dissociated using Accutase to a single cell suspension and transferred to culture dishes coated with fibronectin, collagen IV, laminin or gelatin. Additionally, after being transferred to the different substrata, the cardiosphere-forming cells were divided into two groups: one group cultured without growth factors and a second group in which the culture medium was supplemented with growth factors (Figs. 5-8). Differences in cell morphology between the treatment groups were monitored. After 24 h, the morphology of the cells in the two groups was similar. Phase contrast microscopy revealed spindle-shaped cells, among which single, small, phase-bright, round cells were observed. On the laminin-coated dishes after 24 h, cardiosphere structures were observed in both the growth factor-supplemented and non-supplemented groups. After a period of 8 days, the morphology of the cells in these two groups differed significantly (Fig. 7). With the exception of the fibronectin group, in which the formation of cardiospheres and capillary-like structures was observed in the group cultured with growth factors, spindle-shaped cells and single, small round cells were observed after 8 days of culture with growth factors. In contrast, the cells cultured on the same substratum but without growth factors formed a monolayer of small, round, phase-bright cells (Figs. 5-8). A monolayer of small round cells was also observed in the gelatin-coated dishes cultured with growth factors (Fig. 8).

In the culture of cardiosphere-forming cells cultured with growth factors, the number of cells after 8 days of culturing was nearly the same as after 24 h; no significant subsequent proliferation was observed. In contrast, in the same time period, a significant increase in the number of cells was observed in the group cultured with the same substratum but without growth factors. This result indicates
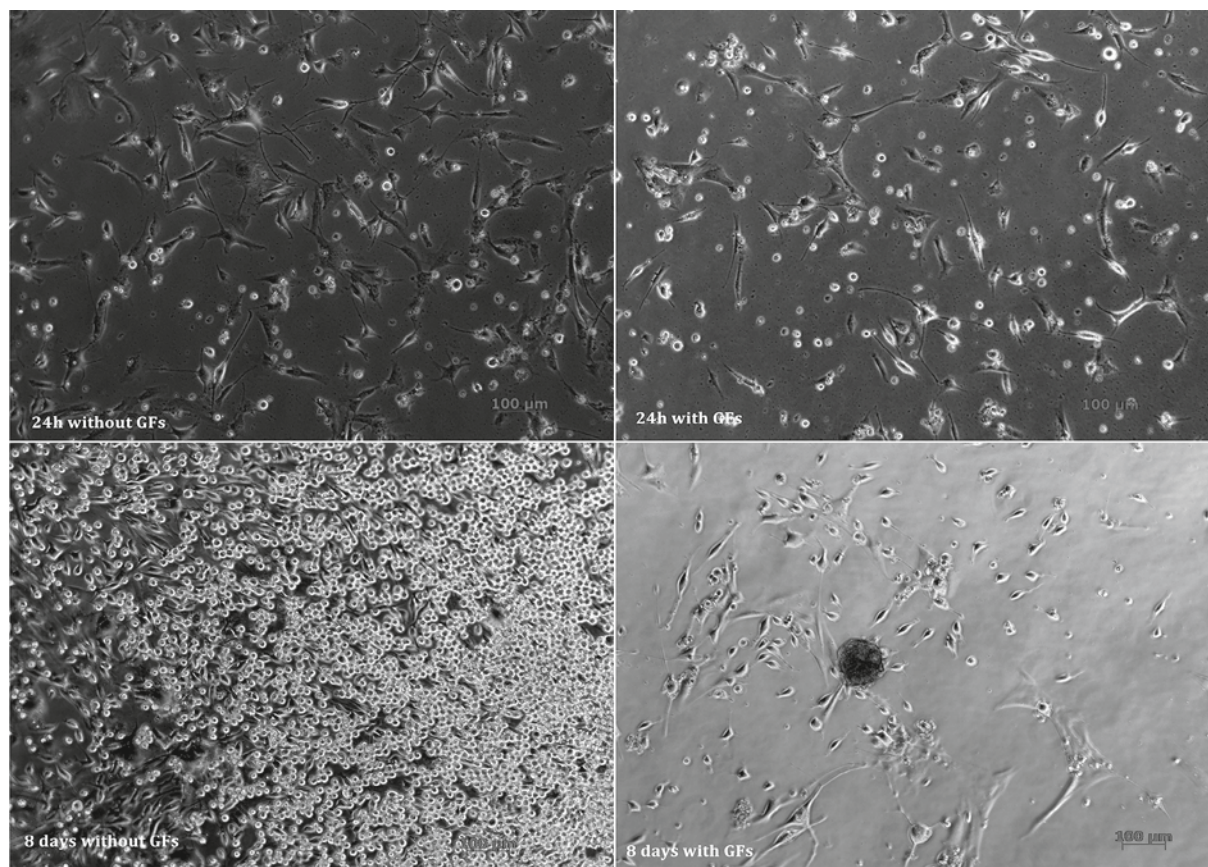

Fig. 5. Effects of fibronectin on CSCs/CPCs cultured without growth factors (GFs) and with GFs at different times
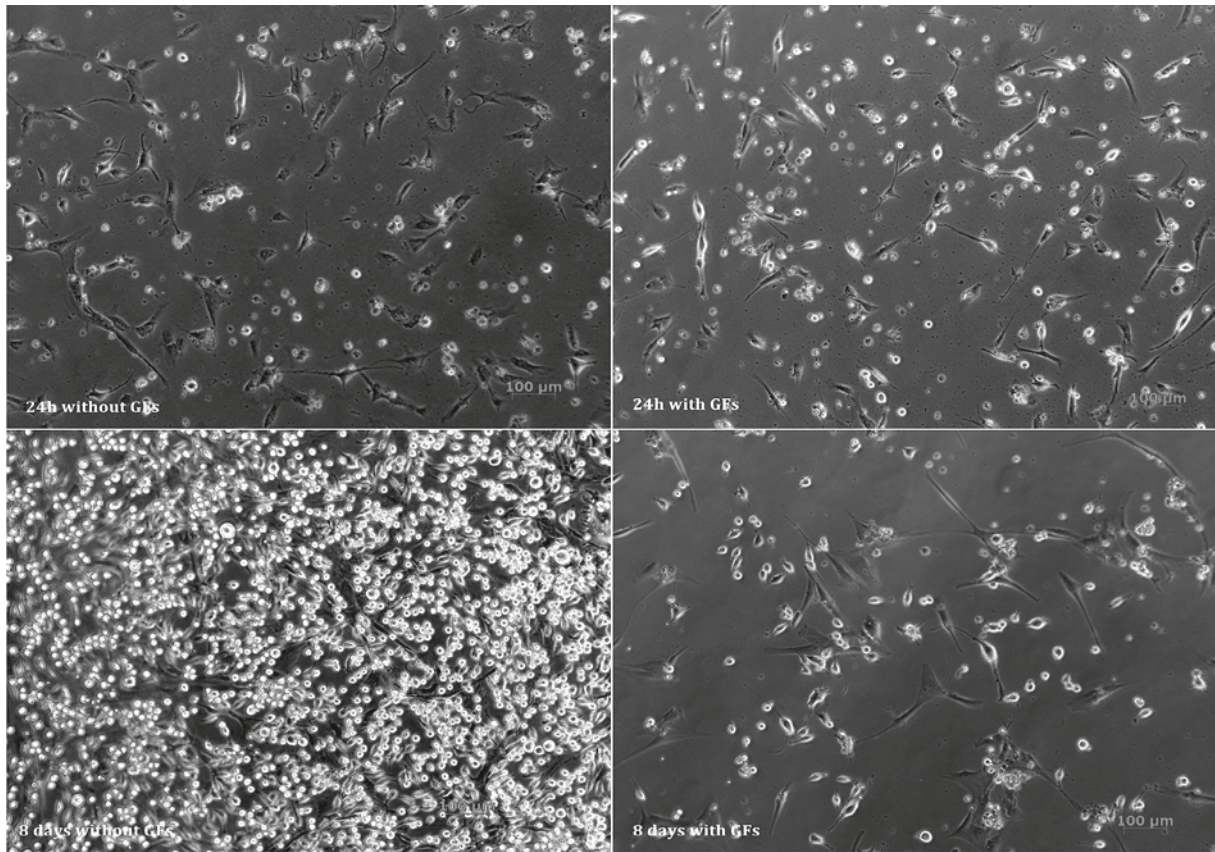
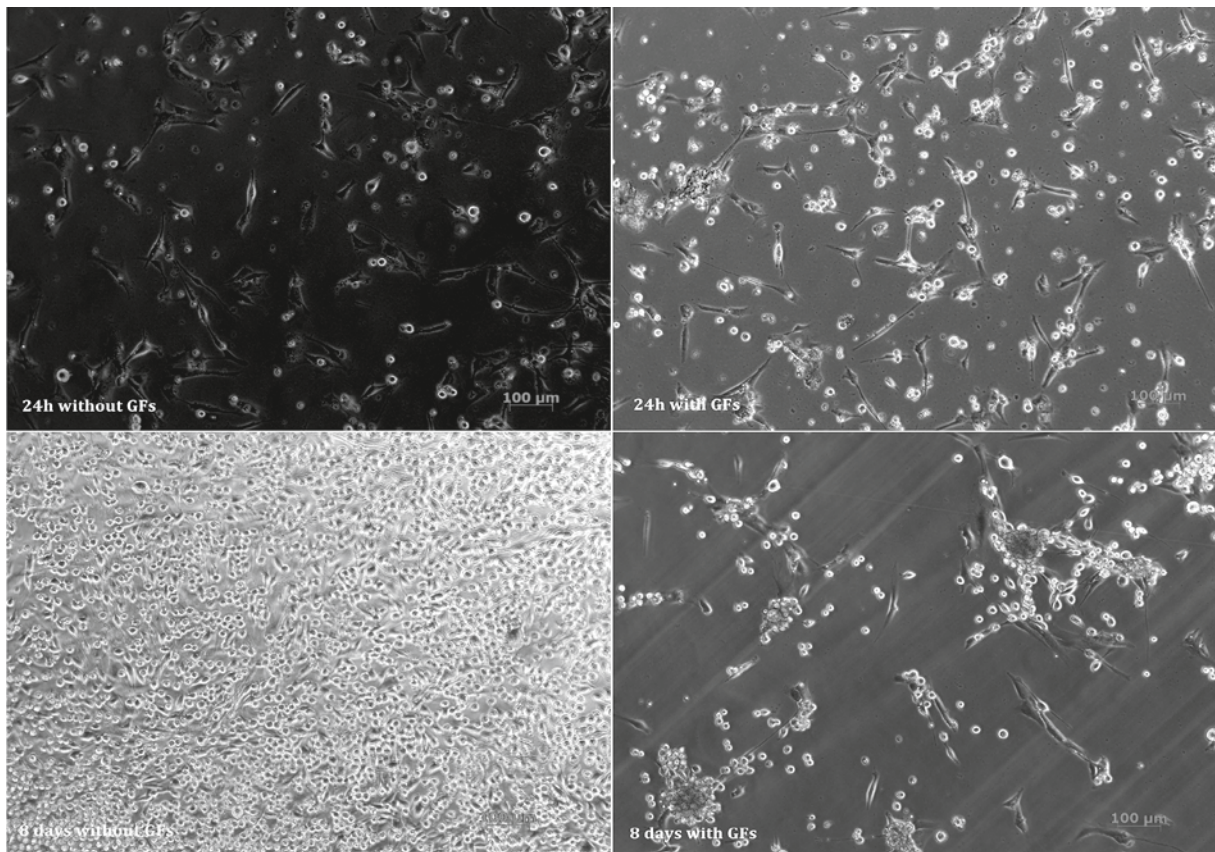**Fig. 6.** Effects of collagen IV on CSCs/CPCs cultured without GFs and with GFs at different times

Fig. 7. Effects of laminin on CSCs/CPCs cultured without GFs and with GFs at different times
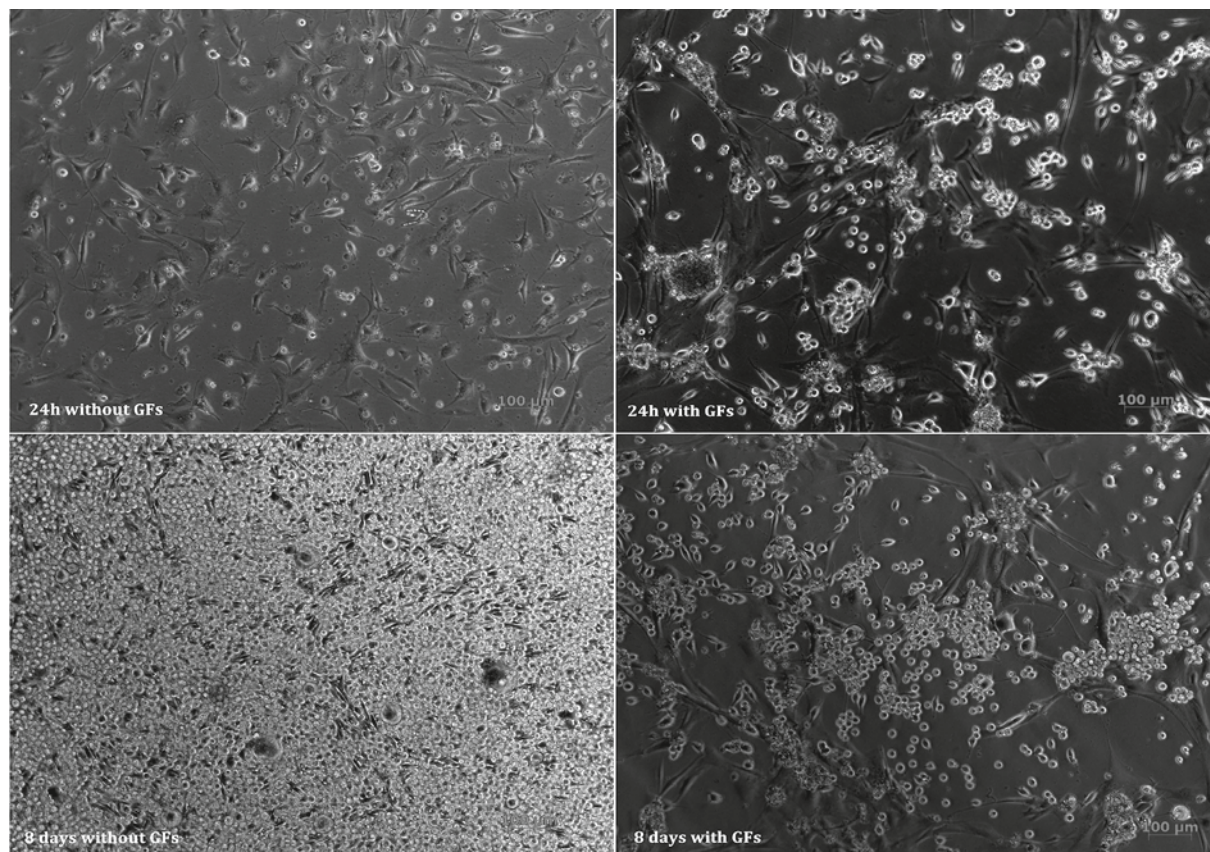
Fig. 8. Effects of gelatin on CSCs/CPCs cultured without GFs and with GFs at different times

a significantly higher proliferation rate in this group than in the group cultured with growth factors.

Fractal dimension analysis

To quantify the complex morphology of the cells in culture, fractal dimension (FD) analysis was performed via the box-counting method. After 24 h of culture, the FD was nearly the same in the cells cultured with growth factors and in those cultured on the same substratum but without growth factors. The initial FD was approximately 1.5 in all of the investigated groups. After 8 days of culturing, the FD increased significantly in the cells cultured without growth factors, reaching a value of approximately 1.8. Thus, the FD increased by approximately 20% (Figs. 9-12). In the sole case of cells cultured on gelatin-coated dishes without growth factors, the FD increased by 10%, as the initial value after 24 h was higher than in the other study groups and the FD was 1.6 (Fig. 12). In the case of cells cultured with growth factors, the FD increased only slightly by approximately 2-5% during the 8 days of culturing (Figs. 9-12). Significant differences between the cells cultured with growth factors and without growth factors were visible starting from day 6 of the culture. This finding may indicate greater complexity of the cultures not supplemented with growth factors.

Percentage of c-kit- and MDR-1-positive cells in cultures on different substrata with and without growth factors

After a period of 8 days, the percentage of c-kit- and MDR-1-positive cells differed significantly depending on the culture conditions. The initial proportions of c-kit- and MDR-1-positive cells in the secondary culture on poly-D-lysine were 6.9% and 4.4%, respectively. In the case of cells cultured without growth factors on collagen IV-coated dishes, the percentage of c-kit-positive cells was 7.1%. This value was not significantly different from that obtained on poly-D-lysine, but the percentage of MDR-1-positive cells was higher on poly-D-lysine, reaching a value of 9%. In the case of cells cultured on laminin- and gelatin-coated dishes, the percentages of both c-kit- and MDR-1-positive cells were lower in relation to the values on poly-D-lysine. Significantly greater percentages of c-kit- and MDR-1-positive cells were observed on the fibronectin-coated dishes, reaching values of approximately 14% for the c-kit receptor and 21% for the MDR-1 receptor (Fig. 13).

In the case of cells cultured with growth factors on collagen IV-, gelatin- and fibronectin-coated dishes, the percentage of cells expressing the c-kit receptor was insignificantly higher than that obtained on poly-D-lysine. In contrast, the c-kit-positive cells in the group of laminin-coated dishes
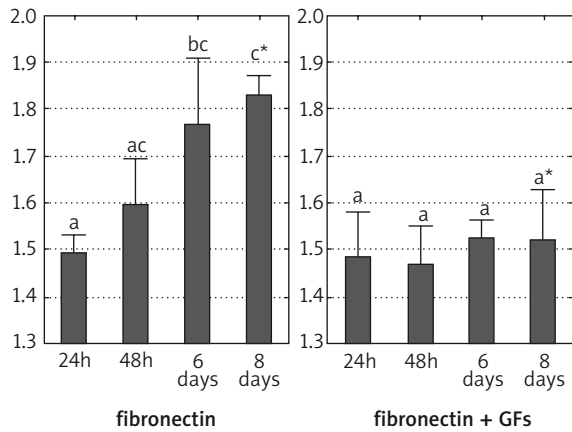
Fig. 9. Time-dependent FD changes in fibronectin coated dishes cultured without GFs and with GFs. Different letters (a, b, c) indicate significant differences between cell culture within the groups cultured without GFs and with GFs according to the time of culture ($x \pm SD$; ANOVA, Tukey test, $p < 0.05$); * indicates significant difference between the groups cultured without GFs and with GFs for the same time of culture ($x \pm SD$; ANOVA, Tukey test, $p < 0.05$)
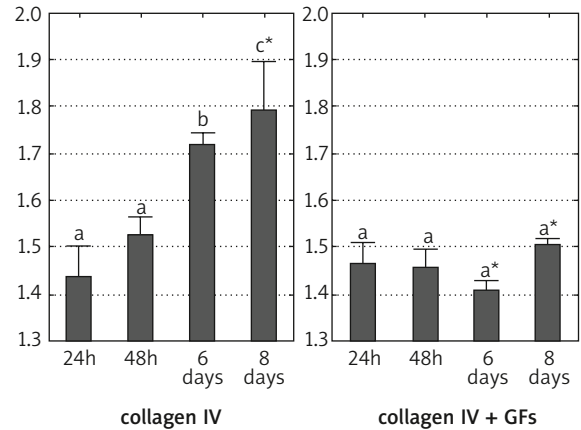
Fig. 10. Time-dependent FD changes in collagen IV coated dishes cultured without GFs and with GFs
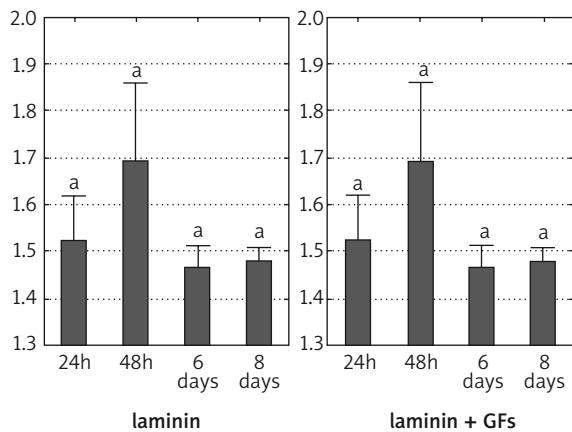
Fig. 11. Time-dependent FD changes in laminin coated dishes cultured without GFs and with GFs
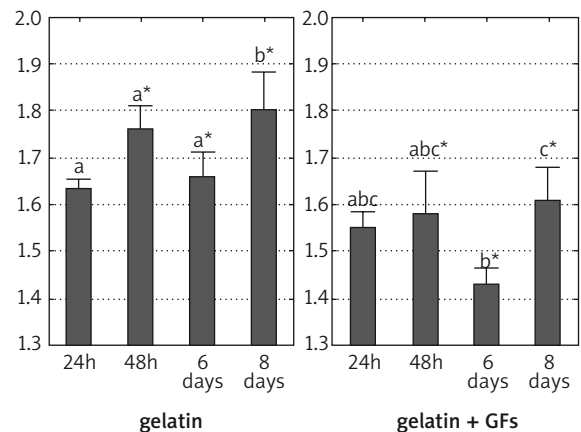

Fig. 12. Time-dependent FD changes in gelatin coated dishes cultured without GFs and with GFs

decreased approximately 2-fold in relation to growth on poly-D-lysine. The percentage of MDR-1 positive cells was approximately 3.2% on collagen IV-coated dishes and approximately 1.8% on laminin-coated dishes, which are values lower than those obtained on poly-D-lysine. Similar values for the MDR-1 receptor were observed for the cells cultured on gelatin- and fibronectin-coated dishes (Fig. 13).

Comparing the cells cultured with growth factors to those cultured without growth factors in collagen-coated dishes, the percentage of c-kit positive cells was roughly the same, but for the MDR-1 receptor, the value obtained for the growth factor group was approximately two-fold lower. In the groups of cells cultured on laminin- and fibronectin-coated dishes without growth factors, higher percentages of c-kit- and MDR-1-positive cells were observed. In contrast, for the gelatin group, higher values for both receptors were observed in the cell cultures supplemented with growth factors.

Discussion

Cells capable of self-renewal and bearing the potential of developmental plasticity have recently been shown to reside in the myocardium. These (CSCs/CPCs) are multipotent and clonogenic, giving rise to cardiomyocytes, smooth muscle cells and endothelial cells both *in vivo* and *in vitro*. The use of CSCs/CPCs in cell therapeutic applications for the reconstruction of ischemically altered heart tissue is very promising. However, the effectiveness of cell therapies strongly depends on methods for administering stem cells to the target area. The type of stem cell transfer depends on many factors, such as the clinical scenario. In the case of acute heart disease, the cells can be administered peripherally or locally through the circulatory system. In chronic ischemia and non-ischemic cardiomyopathy, the preferred method is direct injection into the heart muscle [1]. One of these methods is surgical intramyocardial injection, which can be performed during routine surgical procedu-
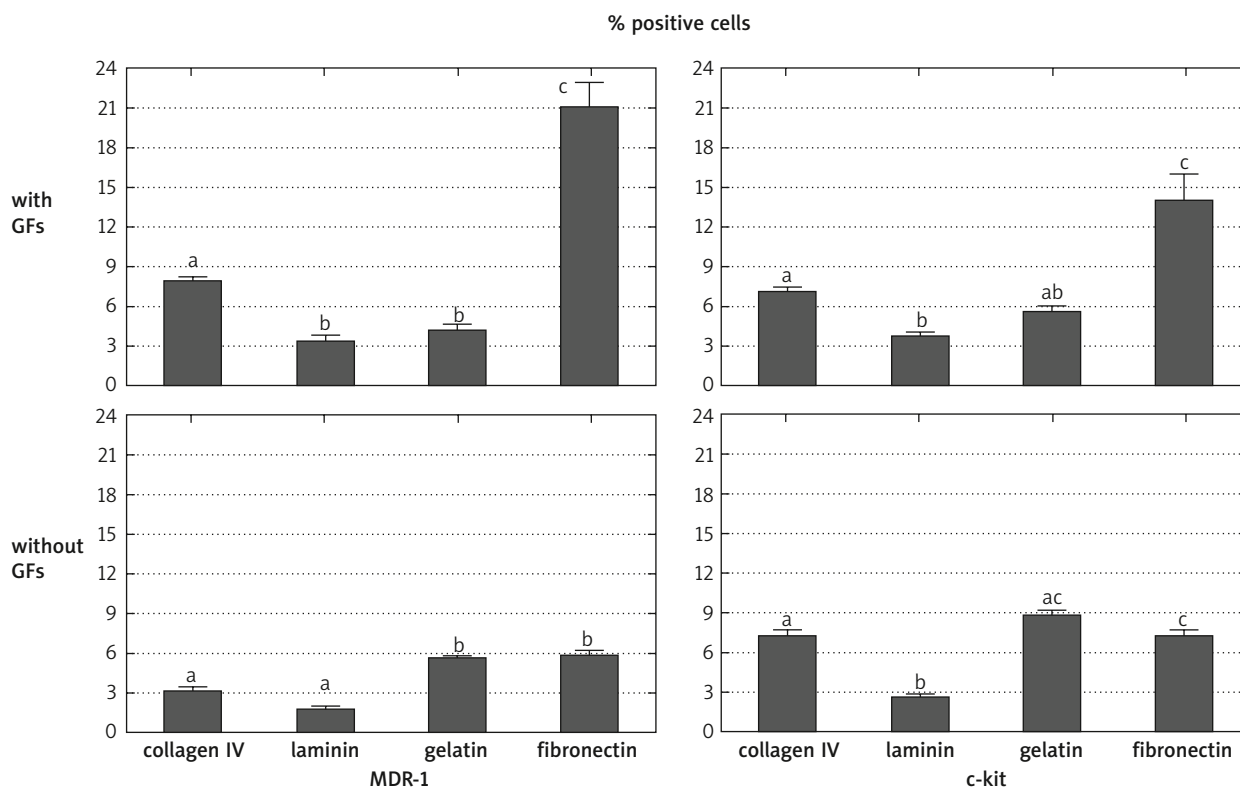
% positive cells

Fig. 13. Flow cytometry analysis of CSCs/CPCs cultured on different substrata without GFs and with GFs – percentage of MDR-1 and c-kit positive cells. Different letters (a, b, c) indicate significant differences between cell culture within the groups cultured without GFs and with GFs

res. The advantages of this type of administration include the ability to directly visualize and inspect the injection area. In a study of Herreros et al. [2], patients with a previous myocardial infarction and ischemic coronary artery disease underwent the injection of skeletal myoblasts during a coronary artery bypass procedure. The authors concluded that the intramyocardial injection of cells combined with a coronary artery bypass is safe; they observed increases in both global and regional left ventricular function and no induction of arrhythmias. The other method of stem cell administration is transendocardial injection. This method utilizes the NOGA system for the precise mapping of the border zone of the infarct [3]. Fuchs et al. observed that the transendocardial injection of bone morrow cells induces endothelial cell proliferation, stimulates angiogenesis and augments the perfusion of ischemic myocardium. Alternatively, stem cell administration can be monitored by MRI, echocardiography or computed tomography. Intracoronary injection of stem cells has been successfully performed in clinical trials. In the study of Strauer et al., autologous mononuclear bone marrow cells were transplanted using a balloon catheter placed in an infarct-related artery. They observed that the intracoronary injection of autologous cells can be safe and clinically effective [4]. The simplest method of stem cell transfer is intravenous injection. Boomsma [5] demonstrated that intravenously injected murine mesenchymal stem cells are able to target the myocardium, attenuate cardiac dysfunction and myocardial remodeling and preserve systolic function. All of these methods of stem cell administration to the infarcted area have some limitations. Intracoronary administration is not suitable for larger cells such as myoblasts because of the risk of embolization of the cells, impaired coronary flow and/or myocardial cell necrosis. With the intravenous method, there is the problem of targeting the cells and that only a small portion of the cells enter the coronary circulation and migrate into the ischemic myocardium [1]. It is also important to note that there are limited data in the literature concerning methods of administering CSCs/CPCs for the treatment of heart disease.

Because of these disadvantages, the optimal route of administering stem cells, such as CSC/CPC administration, is still being sought. One technique that can improve cell therapies and tissue engineering applications is the combination of stem cells with a scaffold composed of natural or synthetic materials. The use of a scaffold for stem cell therapy, including heart disease treatment, is attractive because it harbors the stem cells in an environment that mimics normal cellular conditions. In our study on the culture of cardiac stem/progenitor cells, we used natural components of the extracellular matrix (ECM) as starting points for developing scaffolds based on natural materials. We cultured

the CSCs/CPCs on fibronectin-, collagen IV-, laminin- and gelatin-coated surfaces. We decided to test these factors and their influence on the morphology and phenotype of CSCs/CPCs because the extracellular matrix is composed of three major proteins: fibronectin, collagen and laminin. It is well known that stem cells require the local microenvironment of their niche to develop [6, 7]. The ECM is the main component of the niche, and it is involved not only in mechanically supporting stem cells but also in the regulation of their proliferation, migration and differentiation [8].

In the absence of growth factors, we observed no significant difference in the numbers of c-kit- or MDR-1-positive cells on collagen IV-coated dishes when compared with the results obtained on poly-D-lysine. In the case of cells cultured on laminin or gelatin, a slight decrease in the percentage of c-kit- and MDR-1-positive cells was observed relative to the results obtained on poly-D-lysine. The opposite results were obtained with the fibronectin-coated dishes, on which we observed a roughly three-fold increase in both c-kit- and MDR-1-positive cells. These differences may be caused by the fact that the extracellular matrix components used in the CSC/CPC cultures differ in their structural, biochemical and mechanical properties and that the stem cells react to all these stimuli. In a study by Rowlands et al. [9], when mesenchymal stem cells (MSCs) were cultured on a collagen IV- or laminin-coated surface, only poor induction of differentiation was observed. However, when the MSCs were cultured on a stiffer scaffold with elasticity > 30 kPa and on collagen I or fibronectin coupled to the surface, the production of early osteogenesis markers was observed, suggesting a role in differentiation of the complete environment. The structure of the components can also affect how stem cells differentiate. With a collagen-based structure, MSC osteogenesis is inhibited when the cells are cultured on collagen gel, but this inhibition is not observed when the cells are cultured as a monolayer on collagen-coated dishes [10]. It is interesting that differentiation depends not only on the type of the substratum but also on its mechanical properties, such as elasticity. It was observed that embryoid body formation is inhibited when the collagen gel stiffness is approximately 34 kPa, but when the scaffold is more flexible (16 kPa), inhibition is not observed [11]. In the same study by Battista et al., the induction of endothelial cells was observed when soft collagen was supplemented with fibronectin, but after laminin addition, the embryonic stem cells differentiated into cardiac muscle cells.

The myocardium is composed of cardiac cells placed in the extracellular matrix. As with other types of stem cells, the use of molecules such as collagen, laminin, and fibronectin in *in vitro* cultures can induce the differentiation of adult cardiac stem cells into cardiomyocytes. Moreover, the activation of spontaneous contractility and morphological differentiation into neonatal cardiomyocytes can be observed in cell culture [12]. It has been observed in both *in vitro* and *in vivo* studies that not only separate ECM elements but also a mixture of these components can stimulate the differentiation of stem cells into cardiomyocytes. In a paper by

Bick et al., a beneficial effect of Cardiogel, an ECM mixture composed mainly of laminin and fibronectin, on the differentiated morphology of primary cardiomyocytes was reported [13]. In a study by Leor et al. [14], fetal cardiac myocytes were seeded on an alginate scaffold and cultured for 4 days. This cardiomyocyte-seeded scaffold was implanted into a rat model 7 days after myocardial infarction. They observed that the 3D alginate scaffold provided a good environment for cardiomyocyte growth. After implantation into the infarcted heart, neovascularization of the left ventricle (LV) and attenuation of LV dilatation were observed. In a study by Akhari, heart cells were seeded onto a gelatin matrix and subjected to cyclical mechanical stress using the Bio-Stretch Apparatus. The use of cyclical stretching improved the proliferation and distribution of the seeded cells, and the formation and organization of the matrix were enhanced [15]. Biomaterials used in regenerative medicine applications can serve not only as a scaffold for cell proliferation and differentiation but also to capture stem cells, thereby providing enrichment for autologous cells at the target area. One of these methods conjugates Sca-1 antibody, which specifically recognizes cardiac cells, to a collagen scaffold. This scaffold with a covalently conjugated antibody can effectively regenerate heart tissue when transplanted into the infarcted area [16].

To explain the differences in c-kit- and MDR-1-positive cells observed with different substrata, it is important to note that there are multiple mechanisms involved in the ECM-based control of stem cell fate, including the geometry and elasticity of the ECM and mechanical signal transmission from the ECM to cells [17]. Knowledge regarding these mechanisms is very important for the proper construction of a scaffold on which cardiac stem cells can be seeded, and it is also important for understanding the behavior of cardiac stem cells after implantation into the diseased heart. The influence of ECM stiffness on cell fate was revealed in the study by Emerman et al. [18], in which it was observed that mouse epithelial cells display increased differentiation when cultured on soft collagen compared with the normal plastic surface of culture dishes. In another study, myoblasts cultured on polymer gel developed actin/myosin striations, and this effect was not observed with myoblasts cultured on a collagen surface [19]. In another study by Engler et al., MSCs were cultured on substrates with different stiffnesses; the cells cultured on the soft scaffold differentiated into neurons, those cultured on the intermediate scaffold differentiated into myoblasts, and the stiff substratum promoted the differentiation of MSCs into osteoblasts [20]. The nanotopography of the ECM may be another factor that influences stem cell fate. It seems that these cells have the ability to sense micro- and nanotopographical changes in the ECM and to react to these changes. The effects of tissue-specific matrix orientation on stem cell differentiation have been investigated by Yin et al. They seeded human tendon stem/progenitor cells (hTSPCs) on aligned and randomly oriented poly-L-lactic acid nanofibers. The cells seeded on the aligned fibers were spindle shaped, well oriented and displayed higher expression

of tendon-specific genes compared to the cells seeded on the randomly oriented fibers. In the same study, they observed that the randomly oriented fibers induced osteogenesis but that the aligned fibers hindered this process [21]. Throughout their life, cells are exposed to mechanical stimuli, and it is believed that cells are not passive biomaterials but instead react to mechanical factors, including ECM mechanical stimuli. In response to these forces, cells remodel their cytoskeleton, which is one of the effects of the stem cell differentiation response [17]. In a study by Arnsdof et al. [22], oscillatory fluid flow as a mechanical signal was examined, and the role of this signal on the osteogenic, chondrogenic and adipogenic differentiation of murine mesenchymal stem cells was determined. They hypothesize that isomeric tension within the actin cytoskeleton induced by fluid flow is one of the essential factors involved in differentiation. The authors found that mechanical stimuli upregulated the genes Runx2, Sox9 and PPARγ, which are critical for cell differentiation. In a study by Gong [23], MSCs were cultured on different protein-coated flexible membranes. When they additionally exposed the cells to 5% or 10% cyclic uniaxial stretching, the myogenic phenotype and the expression of smooth muscle actin (SMA) were observed.

An important factor involved in the regulation of cell fate (including stem cell fate) is cell shape. Changes in shape may be involved in myocardial development [24]. It was observed by Holzer et al. [25] that chondrocytes cultured in flattened 2D conditions dedifferentiate and adopt a fibroblastic morphology. The same cells cultured in 3D conditions in agarose or alginate gel retain normal chondrocyte morphology [26]. Because of this, in our study, the influences of different substrata, including fibronectin, collagen IV, laminin and gelatin, on the changes in cell shape were analyzed. Additionally, the effect of growth factor supplementation on cell morphology was analyzed. The tools available for analyzing cell morphology and cell shape in populations of *in vitro* cultured cells are not ideal. As an attractive alternative, fractal analysis can be used to quantify changes in the morphology of *in vitro* cultured cells. Limited data exist regarding the morphology and shape of cardiac stem cells cultured on different substrata in different conditions. In our study, we monitored the morphology of cells cultured on different substrata both with and without growth factor supplementation. The general tendency observed was that the FD of the cells increased significantly (by approximately 20%) in the secondary cultures of CSCs/CPCs cultured without growth factors, and in the cells cultured with growth factors, there was only a slight increase (approximately 4%) in the FD value. In a study by Pearson [27], image and fractal analyses were used to determine how the viscosity of the culture medium affects osteoblast morphology. In that study, cells were grown in culture medium supplemented with methyl cellulose (MeC). The authors demonstrated that the medium viscosity can affect cell morphology and that fractal analysis can be used to qualitatively analyze these changes and to classify the cells based on morphology. In a paper by Bernard [28], Mandelbrot's fractal dimension was used to quantify the morphology of glial cells. They observed that when the morphological complexity of the cells increases, their FD value increases, and it was hypothesized that the FD value can be used to determine the stage of differentiation. In another experiment, they calculated the FD value for rat cerebellar oligodendrocytes during differentiation in primary culture. These data were correlated with the expression of A2B5, O4 and anti-galactocerebroside (Gal-C), which are commonly used differentiation markers. Their results indicate that the FD value can be used to determine the stage of differentiation.

In our study, we also observed trends involving the FD value and the percentages of c-kit- and MDR-1-positive cells. When the FD value increased during a day of culturing, increases in c-kit- and MDR-1-positive cells were also observed. An increase in the FD indicates an increase in the complexity of the cell culture. Additionally, in conjunction with phenotypic calibration, FD values can allow the stage of cardiac stem cell differentiation to be determined, which can be useful in the construction of a dedicated scaffold for use as a cell delivery system in the treatment of diseased hearts.

Because of their potential clinical applications, the ideal culture medium for the growth, proliferation and differentiation of cardiac stem cells should be serum-free and supplemented with specific growth factors. However, cells cultured in serum-free media display a lower mitotic index, whereas apoptosis and pure adhesion are stimulated [29]. Because of this, it is important to supplement the culture medium with growth factors that promote the proliferation and growth of the cells. Depending on the final application, the growth factors chosen can promote the differentiation or expansion of the stem cells.

In our study, we cultured CSCs/CPCs on different substrata in culture medium without growth factors and on the same substratum but in culture medium supplemented with 20 ng/ml bFGF, 2% B27, 4 ng/ml cardiotrophin, 10 ng/ml EGF, 40 nM/l thrombin, and 0.2% mercaptoethanol. We observed that with the collagen IV-, laminin- and fibronectin-coated dishes, the groups supplemented with growth factors presented fewer c-kit- and MDR-1-positive cells than the groups cultured without growth factors. Specifically, significant differences were observed between the fibronectin groups, in which approximately two-fold and four-fold fewer c-kit- and MDR-1-positive cells, respectively, were observed. Only for the laminin substrate was a slight increase in the percentage of c-kit- and MDR-1-positive cells observed in cultures supplemented with growth factors. This result indicates that although the use of growth factors can induce the differentiation of CSCs/CPCs, to expand the number of undifferentiated c-kit-positive cardiac stem cells, culturing without growth factors is the preferred method.

The study by Urbanek et al. revealed that most CSCs express c-Met, insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF). After injecting IGF-1 and HGF into infarcted hearts, mobilization of CSCs to the damaged area was observed, and the mobilized CSCs grew, survived and proliferated [30]. HGF promotes cell migration [31]. IGF-1

acts as an antiapoptotic and mitotic factor in the infarcted myocardium, where it promotes myocyte formation and the proliferation and survival of cardiac cells [32]. Additional growth factors involved in normal heart development are a part of the fibroblast growth factor (FGF) family. In a study by Chan et al., embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells) were cultured with the growth factors FGF-2, FGF-3, FGF-6, FGF-7, FGF-10 and FGF-15. They observed an impact of FGF-10 on differentiation into cardiomyocytes for both ES and iPS cells [33].

In another study, when ES cells were cultured with bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and dickkopf homolog 1 (DKK1) in serum-free medium, a KDR^{low}/ $C-KIT(CD117)^{neg}$ population was generated that can differentiate into cardiac, endothelial and vascular smooth muscle cells *in vitro* [34]. Another factor used in the culture of CSCs/ CPCs is epidermal growth factor (EGF). EGF interacts with the adrenergic receptor to decrease the response to epinephrine, and EGF can protect the heart from the harmful effects of epinephrine [35]. In an *in vitro* study, it was observed that in cultures of ventricular cardiomyocytes and noncardiomyocytes from neonatal rat hearts, EGF increased cAMP accumulation in a time-dependent manner in the cardiomyocytes but not in the noncardiomyocytes [36]. In the study by Messina, cardiac stem cells were cultured on poly-D-lysine-coated dishes and supplemented with thrombin. During the first week of growth, this factor led to a 7-fold increase in the number of cardiospheres [37], but the mechanism by which the number of spheres increased in the culture remained unclear. One mechanism that can explain the role of thrombin in cell cultures is that thrombin can stimulate nuclei to re-enter S-phase via the phosphorylation of retinoblastoma protein [38]. An important factor that can be used when culturing adult cardiac stem cells is cardiotrophin-1 (CT-1). Cardiotrophin-1 is a member of the IL-6 family of cytokines, which are expressed in the adult heart, where they induce hypertrophy and enhance the survival of cardiomyocytes. As described by Chen, CT-1 has an effect on the differentiation of bone marrow mesenchymal stem cells (BMMSCs). Cultures of BMMSCs induced with 5-azacytidine (5-aza) and supplemented with CT-1 were found to differentiate into cardiomyocytes. In that study, the expression of GATA-4, Nkx-2.5, alpha-actin and beta-MHC mRNAs was observed, confirming the role of CT-1 in the differentiation and maturation of cardiomyocyte-like cells. In another study [39], a protective effect of CT-1 against hypoxia/ischemia was observed both *in vitro* and *in vivo*. Cultured adult rat cardiocytes were examined, cardiac cells were exposed to hypoxia followed by reoxygenation, and CT-1 was administered prior to hypoxia. CT-1 was found to have a protective effect, reducing ischemic damage in the cultured cells.

Conclusion

The proper growth, migration and differentiation of cardiac stem cells depends on many factors, such as the interaction of the cells with extracellular matrix components, which act not only as a support for the cultured cells but also actively influence their proliferation, migration, adhesion and differentiation. These parameters should be considered in the construction of a scaffold material to be used for the effective delivery of cardiac stem cells to the damaged heart. Additionally, the use of an appropriate combination of growth factors is another important element in directing cardiac stem cells to differentiate into cardiomyocytes, endothelial cells or vascular smooth muscle cells. From the clinical point of view, it is important to select an appropriate cell therapy strategy. Most papers have focused on the use of undifferentiated cells, but it is possible that some degree of differentiation is beneficial for certain stem cell therapy applications.

Acknowledgements

References

1. Perin EC, Geng YJ, Willerson JT. Adult stem cell therapy in perspective. Circulation 2003; 107: 935-938.
2. Herreros J, Prósper F, Perez A, Gavira JJ, Garcia-Velloso MJ, Barba J, Sánchez PL, Cañizo C, Rábago G, Martí-Climent JM, Hernández M, López-Holgado N, González-Santos JM, Martín-Luengo C, Alegria E. Autologous intramyocardial injection of cultured skeletal muscle-derived stem cells in patients with non-acute myocardial infarction. Eur Heart J 2003; 24: 2012-2020.
3. Fuchs S, Baffour R, Zhou YF, Shou M, Pierre A, Tio FO, Weissman NJ, Leon MB, Epstein SE, Kornowski R. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 2001; 37: 1726-1732.
4. Strauer BE, Brehm M, Zeus T, Köstering M, Hernandez A, Sorg RV, Kögler G, Wernet P. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002; 106: 1913-1918.
5. Boomsma RA, Swaminathan PD, Geenen DL. Intravenously injected mesenchymal stem cells home to viable myocardium after coronary occlusion and preserve systolic function without altering infarct size. Int J Cardiol 2007; 122: 17-28.
6. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature 2001; 414: 98-104.
7. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. Cell 2004; 116: 769-778.
8. Hayashi Y, Furue MK, Okamoto T, Ohnuma K, Myoishi Y, Fukuhara Y, Abe T, Sato JD, Hata R, Asashima M. Integrins regulate mouse embryonic stem cell self-renewal. Stem Cells 2007; 25: 3005-3015.
9. Rowlands AS, George PA, Cooper-White JJ. Directing osteogenic and myogenic differentiation of MSCs: interplay of stiffness and adhesive ligand presentation. Am J Physiol Cell Physiol 2008; 295: C1037-C1044.
10. Lund AW, Stegemann JP, Plopper GE. Inhibition of ERK promotes collagen gel compaction and fibrillogenesis to amplify the osteogenesis of human mesenchymal stem cells in three-dimensional collagen I culture. Stem Cells Dev 2009; 18: 331-341.
11. Battista S, Guarnieri D, Borselli C, Zeppetelli S, Borzacchiello A, Mayol L, Gerbasio D, Keene DR, Ambrosio L, Netti PA. The effect of matrix composition of 3D constructs on embryonic stem cell differentiation. Biomaterials 2005; 26: 6194-6207.
12. Hilenski LL, Terracio L, Borg TK. Myofibrillar and cytoskeletal assembly in neonatal rat cardiac myocytes cultured on laminin and collagen. Cell Tissue Res 1991; 264: 577-587.

13. Bick RJ, Snuggs MB, Poindexter BJ, Buja LM, Van Winkle WB. Physical, contractile and calcium handling properties of neonatal cardiac myocytes cultured on different matrices. Cell Adhes Commun 1998; 6: 301-310.

14. Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S. Bioengineered cardiac grafts: a new approach to repair the infarcted myocardium? Circulation 2000; 102: (19 Suppl 3): III56-III61.

15. Akhyari P, Fedak PW, Weisel RD, Lee TY, Verma S, Mickle DA, Li RK. Mechanical stretch regimen enhances the formation of bioengineered autologous cardiac muscle grafts. Circulation 2002; 106 (12 Suppl 1): I137-I142.

16. Shi C, Li Q, Zhao Y, Chen W, Chen B, Xiao Z, Lin H, Nie L, Wang D, Dai J. Stem-cell-capturing collagen scaffold promotes cardiac tissue regeneration. Biomaterials 2011; 32: 2508-2515.

17. Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 2009; 5: 17-26.

18. Emerman JT, Burwen SJ, Pitelka DR. Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue Cell 1979; 11: 109-119.

19. Engler AJ, Griffin MA, Sen S, Bönnemann CG, Sweeney HL, Discher DE. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. J Cell Biol 2004; 166: 877-887.

20. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell 2006; 126: 677-689.

21. Yin Z, Chen X, Chen JL, Shen WL, Hieu Nguyen TM, Gao L, Ouyang HW. The regulation of tendon stem cell differentiation by the alignment of nanofibers. Biomaterials 2010; 31: 2163-2175.

22. Arnsdorf EJ, Tummala P, Kwon RY, Jacobs CR. Mechanically induced osteogenic differentiation-the role of RhoA, ROCKII and cytoskeletal dynamics. Journal of Cell Science 2009; 122: 546-553.

23. Gong Z, Niklason LE. Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs). FASEB J 2008; 22: 1635-1648.

24. Manasek FJ, Burnside MB, Waterman RE. Myocardial cell shape change as a mechanism of embryonic heart looping. Dev Biol 1972; 29: 349-371.

25. Holtzer H, Abbott J, Lash J, Holtzer S. The loss of phenotypic traits by differentiated cells in vitro, I. Dedifferentiation of cartilage cells. Proc Natl Acad Sci U S A 1960; 46: 1533-1542.

26. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982; 30: 215-224.

27. Pearson M, Landini G, Shelton RM. Image and fractal analysis of osteoblastic cells in viscous media. J Mater Sci Mater Med 1999; 10: 761-765.

28. Bernard F, Bossu JL, Gaillard S. Identification of living oligodendrocyte developmental stages by fractal analysis of cell morphology. J Neurosci Res 2001; 65: 439-445.

29. Wong M, Tuan RS. Nuserum, a synthetic serum replacement, supports chondrogenesis of embryonic chick limb bud mesenchymal cells in micromass culture. In Vitro Cell Dev Biol Anim 1993; 29A: 917-922.

30. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, Hosoda T, Chimenti S, Baker M, Limana F, Nurzynska D, Torella D, Rotatori F, Rastaldo R, Musso E, Quaini F, Leri A, Kajstura J, Anversa P. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ.Res 2005; 97: 663-673.

31. Powell EM, Mars WM, Levitt P. Hepatocyte growth factor/scatter factor is a motogen for interneurons migrating from the ventral to dorsal telencephalon. Neuron 2011; 30: 79-89.

32. Li Q, Li B, Wang X, Leri A, Jana KP, Liu Y, Kajstura J, Baserga R, Anversa P. Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. J Clin Invest 1997; 1991-1999.

33. Chan SS, Li HJ, Hsueh YC, Lee DS, Chen JH, Hwang SM, Chen CY, Shih E, Hsieh PC. Fibroblast growth factor-10 promotes cardiomyocyte differentiation from embryonic and induced pluripotent stem cells. PLoS ONE 5: e14414.

34. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 2008; 453: 524-528.

35. Lorita J, Escalona N, Faraudo S, Soley M, Ramírez I. Effects of epidermal growth factor on epinephrine-stimulated heart function in rodents. Am J Physiol Heart Circ Physiol 2002; 283: H1887-H1895.

36. Yu Y, Nair BG, Patel TB. Epidermal growth factor stimulates cAMP accumulation in cultured rat cardiac myocytes. J Cell Physiol 1992; 150(3) 559-567.

37. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004; 95: 911-921.

38. Tanaka EM, Drechsel DN, Brockes JP. Thrombin regulates S-phase re-entry by cultured newt myotubes. Curr Biol 1999; 9: 792-799.

39. Liao Z, Brar BK, Cai Q, Stephanou A, O'Leary RM, Pennica D, Yellon DM, Latchman DS. Cardiotrophin-1 (CT-1) can protect the adult heart from injury when added both prior to ischaemia and at reperfusion. Cardiovasc Res 2002; 53: 902-910.