Behavior of Cardiomyocytes and Skeletal Muscle Cells on Different Extracellular Matrix Components—Relevance for Cardiac Tissue Engineering

Karin Macfelda, Barbara Kapeller, Ingrid Wilbacher, and Udo M. Losert

Core Unit for Biomedical Research, Medical University Vienna, Vienna, Austria

**Abstract:** Myocardial cell transplantation in patients with heart failure is emerging as a potential therapeutic option to augment the function of remaining myocytes. Nevertheless, further investigations on basic issues such as ideal cell type continue to be evaluated. Therefore, the aim of our studies was to compare the performance of skeletal muscle cells and cardiomyocytes with respect to their proliferation rate and viability on different extracellular matrix components (EMCs). Rat cardiomyocytes (RCM) and rat skeletal muscle cells (RSMC) were cultured on EMCs such as collagen type I, type IV, laminin, and fibronectin. The components were used as “single coating” as well as “double coating.” Proliferation rates were determined by proliferation assays on days 1, 2, 4, and 8 after inoculation of the cells. The most essential result is that collagen type I enhances the proliferation rate of RSMC but decreases the proliferation of RCM significantly. This effect is independent of the second EMC used for the double-coating studies. Other EMCs also influence cellular behavior, whereas the sequence of the EMCs is essential. Results obtained in our studies reveal the significant different proliferation behavior of RCM and RSMC under identical conditions. As skeletal muscle cells are also used in heart tissue engineering models, these results are essential and should be investigated in further studies to prove the applicability of skeletal muscle cells for heart tissue engineering purposes.

**Key Words:** Cardiomyocytes—Skeletal muscle cells—In vitro—Extracellular matrix—Tissue engineering.

Cardiac tissue engineering and myocardial cell transplantation for patients with congestive heart failure due to ischemic heart diseases or dilated cardiomyopathies is emerging as a potential therapeutic option to augment the contractile function of the failing heart. Primarily for practical reasons, autologous skeletal myoblasts have been the first to undergo clinical trials, but other cell types are also considered, particularly bone marrow stem cells that are attractive because of their autologous origin and their purported cardiomyocyte transdifferentiation potential in response to cues present in the target organ (1). Recent data on the implantation of differentiated cardiac and noncardiac cells as well as on adult stem cells of different origin have provided hope for the replacement of cells after the irreversible loss of viable cardiac cells that occur during heart failure (1,2).

As compared with the direct injection of a cell suspension into the myocardium, cardiac tissue engineering strategies are now emerging as another form of therapy of the failing heart. For applying tissue engineering methods, the creation of suitable three-dimensional matrices composed of natural or synthetic scaffold materials that host the cells to allow maintenance of cellular viability and differentiation and favor cell integration is a rather critical step (3).

In general, tissue engineering approaches typically apply exogenous three-dimensional extracellular matrices (ECMs) to generate new natural tissues from isolated cells. For this purpose, synthetic ECMs provide an adhesion substrate for transplanted cells and serve as a delivery vehicle into specific sites in the body (4). One approach to designing exogenous ECMs for tissue engineering is to mimic the function of ECM molecules naturally found in tissues as the...
native ECMs act as a scaffold to bring cells together, to control tissue structure and to regulate the cell phenotype (5).

In the heart, the ECM is important for both myocardial structure and function, and plays a critical role in the growth, division, and differentiation of cardiomyocytes (6). The cardiac ECM consists of three-dimensional interstitial collagens—predominantly collagen types I and III—to which other matrix components are attached (7). Cardiomyocytes are surrounded by a basement membrane consisting collagen type IV, laminin, fibronectin, and several proteoglycans, all of which they normally synthesize. Collagen types I and III maintain structural integrity of the myocytes. Other components such as fibronectin and laminin also mediate important functions such as healing and remodeling (7).

In healthy hearts, three major components work in concert to regulate cellular and extracellular remodeling events. These are ECM, integrins on the cell surface, and matrix metalloproteinases. The interplay between these elements regulates myocardial structure and function under normal conditions and in response to stress. Under normal conditions, synthesis and degradation of ECM is a tightly regulated process. During remodeling processes, however, an increase in synthesis and/or decrease in degeneration of ECM results in fibrosis. Fibrosis is defined as the inappropriate wound remodeling leading to pathophysiological accumulation of connective tissue, primarily collagen (6).

Although many researchers are working in the field of cardiac tissue engineering (8–10), the survival of the implanted cells under ischemic conditions present in the injured heart is still a major challenge that will have to be addressed. For an optimal achievement of implanted cells, further investigations may be necessary to gain detailed insight on basic issues including the optimization of cell survival with regard to cell proliferation and differentiation, as well as the choice of the optimal cell type and scaffolds for cardiac tissue engineering purposes.

Researches into the influence of extracellular matrix components (EMCs) on the survival of cardiomyocytes in vitro have been carried out by Lundgren et al. in the 1990s (11–20). Investigations dealt with adhesion and attachment of cardiomyocytes to EMCs but not with cellular behavior to proliferation.

Therefore, the aim of our studies was to investigate the behavior of cardiomyocytes on different EMCs (collagen types I and IV, laminin, and fibronectin), either isolated or in combination, with respect to cell viability and proliferation rate.

**Materials and Methods**

In the present study, cardiomyocytes (rat cardiomyocytes [RCMs]) and skeletal muscle cells (skeletal muscle cells [RSMCs]) of Rattus norvegicus (R. norv.) were used in proliferation assays to evaluate the different behaviors on various EMCs such as collagen types I and IV, laminin, and fibronectin.

**Cell culture**

Established cell lines were obtained from American Type Culture Collection (Manassas, VA, USA): CRL-1446 (H9c2[2–1]) of R. norv. heart tissue origin and CRL-1458 (L6) of R. norv. thigh muscle origin.

Cells were cultivated in Dulbecco minimal essential medium (Gibco, Vienna, Austria) containing 10% fetal calf serum (PAA, Linz, Austria) as well as 100-U/mL penicillin, 100-µg/mL streptomycin (both obtained from Invitrogen, Lofer, Austria), 0.05-mg/mL ascorbic acid (Sigma, St. Louis, MO, USA).

Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cultures were examined daily by phase contrast microscopy (Olympus, Vienna, Austria) for evidence of attachment and viability. Culture medium was changed twice a week. Adherent cells approaching confluence were harvested with 0.5-g/L trypsin—0.2-g/L ethylenediamine tetraacetic acid (Invitrogen) and were subcultivated with a split ratio of 1:3.

For test implementation, RCMs and RSMCs were harvested by trypsinization, centrifuged at 1200 rpm for 10 min (RCF = 300) at room temperature (RT) (22°C), and 3 ¥ 10³ cells were seeded per well (0.32 cm², 96-well plate) in triplicates for each test line.

**Cell characterization**

Cultured cells were stained immunohistochemically for cardiomyocyte specific markers antitroponin T-C (Santa Cruz, Santa Cruz, CA, USA), antimyocardial muscle-actin (Dako A/S, Glostrup, Denmark), as well as myocyte specific markers antimuscle actin (clone HHF 35) and antinmyosin heavy chain (all obtained from Dako A/S). To verify the purity of cell cultures, additional staining against fibroblast antigens (clone 1B10, Harlan Serlab, Leicestershire, UK) was carried out. Anti-IgG (Dako A/S) combined with diamidino-phenylindole (DAPI) used as cell nucleus staining served as negative control.

Cells were cultured on chamber slides (Sigma), washed three times with Tris(hydroxymethyl)aminomethane (Tris) buffered saline (TBS) (0.05-M Tris, 0.15-M NaCl, pH 7.2–7.6), and fixed with precooled acetone at +4°C for 10 min. Next, the cells were...
exposed to primary antibodies against troponin T-C, myocardial muscle actin, muscle actin, myosin heavy chain, fibroblast specific antigens, and IgG overnight at +4°C. All antibodies were used at a dilution of 1:50. After washing the cells with TBS, secondary antibodies (goat-antimouse Alexa Fluor 488 and goat-antirabbit Alexa Fluor 568, Molecular Probes, Leiden, The Netherlands) were added, followed by incubation for 2 h at RT. Finally, DAPI (Sigma) was added for 5 min at RT. Specimens were mounted and coverslipped using fluorescence mounting medium (Dako S/A).

The cells were analyzed with a fluorescence microscope BX 60 (Olympus) and image analysis software (SIS, Muenster, Germany).

Coating of cell culture plates

The surfaces of 96-well plates (Corning, Inc., Corning, NY, USA) were left uncoated or coated with collagen type I (5 µg/cm², Roche, Vienna, Austria), collagen type IV (10 µg/cm², Sigma), laminin (1 µg/cm², Sigma), and fibronectin (5 µg/cm², Chemicon, Temecula, CA, USA) for 1 h at RT. For double-coating experiments, the culture plates were first coated with one coating substance for 1 h at RT, carefully rinsed with phosphate buffered saline (Invitrogen), and then coated with the second coating protein for 1 h at RT.

Proliferation assay

Proliferation assays were performed 1, 2, 4, and 8 days after seeding using an EZ4U kit (Biomedica, Vienna, Austria), which is based upon the use of tetrazolium salts as indicators of cell viability: yellow colored tetrazolium compound is converted to its red formazan derivative. Twenty microliters of dye solution was added to 200 µL of sample. After incubation for 2 h at +37°C, absorbance was measured in optical density units by an E960 microplate reader (Meta-tech, Taibei Hasien, Taïwan) at 450 nm with a 600-nm reference beam to correct nonspecific background values that might be caused by cell debris.

Cell morphology and density analysis

Cell morphology in coated wells was documented 1, 2, 4, and 8 days after preparation. The characteristics, observed through the light microscope, included cell shape, dispersion, and creation of mono- and multilayer (Fig. 1A–J). Additionally, absolute cell numbers were evaluated (Tables 1 and 2).

Cell viability assay

Cell viability was assayed at cell seeding and at day 8 after cultivation. To determinate cell viability, 50 µL was taken from each cell culture and was stained with 0.4% trypan blue (Sigma). The cells were counted with a Bürker–Türck hemocytometer.

Statistical analysis

Mean values and SDs of cell proliferation obtained within six test series were analyzed at defined points of time (1, 2, 4, and 8 days after cell seeding). Data were analyzed using analysis of variance and Leven test. P values less than 0.05 were considered significant. Significance was interpreted as differences in cell proliferation rates on different EMCs.

RESULTS

We verified that all cell cultures consisted of >90% RCMs and RSMCs, respectively, as determined by immunostaining for cardiac specific markers (troponin T-C, myocardial muscle actin) and skeletal muscle cell specific markers (muscle actin, myosin heavy chain) IgG combined with DAPI served as negative control (Fig. 2).

Cell morphology and distribution

Compared to cells grown on uncoated plastic (Fig. 1A,F) cell morphology, density and distribution differed noticeably for both cell types (RCMs in Fig. 1B–E and RSMCs in Fig. 1G–J). Cell size and morphology remained unaffected. The cell shape was not influenced by growing on coated culture surfaces (Fig. 1B–E,G–J) compared to the control (Fig. 1A,F).

The most diametrically opposed trend could be documented for RCMs and RSMCs grown for 8 days on collagen type I-coated wells. Whereas establishment of a proliferative RCMs stratum on collagen type I failed almost completely (Fig. 1B), collagen type I showed the best performance for RSMCs by forming multilayer sheets with numerous interconnected cells in the state of proliferation (Fig. 1G). Grown on collagen type IV, an analogous trend could be documented for both cell types: cell density and distribution increased in comparison to uncoated surfaces, but proliferation rates were quite lower (Fig. 1B–E,G–J) compared to the control (Fig. 1A,F).

Grown on laminin, a quite different growth pattern could be demonstrated for RSMCs and RCMs. Cardiomyocytes showed a very moderate increase in cell number. The cells were distributed in monolayers (Fig. 1D). Skeletal muscle cells in contrast showed a very dense multilayer, which means that the cells grew in multiple superposed layers (Fig. 1I). On fibronectin-coated surfaces, RCMs' best performance was observed. Cellular multilayers were formed and the cells showed interconnections (Fig. 1E). RSMCs...
FIG. 1. Phase contrast micrographs (×100) of cardiomyocyte (RCM)-seeded wells with varying ECM-coatings 8 days after culture. Control—uncoated surface (A), collagen type I (B), collagen type IV (C), laminin (D), fibronectin (E). Phase contrast micrographs (×100) of skeletal muscle cell (RSMC)-seeded wells with varying ECM coatings 8 days after culture. Control—uncoated surface (F), collagen type I (G), collagen type IV (H), laminin (I), fibronectin (J).
Cell viability

The initial value of cell viability at seeding was 98.5% (RCM) and 100% (RSMC), respectively.

RCMs cultured on fibronectin as well as RSMCs developed cell aggregates, and isolated cellular interconnections could be seen (Fig. 1K).

Cell proliferation

To determine the effect of ECM components on proliferation, RCMs and RSMCs were grown on collagen types I and IV, laminin, and fibronectin.

From days 1–4 of culture on exogenous ECM, proliferation rates of RCMs remained more or less unchanged compared to control cells grown on uncoated plastic (Fig. 3A–C). On day 8, collagen type I caused a moderate decrease of proliferation, while...
collagen type IV, laminin, and fibronectin coatings resulted in a significant increase of proliferation rates (Fig. 3D).

RSMCs demonstrated evidence of proliferation in the presence of collagen types I and IV, laminin, and fibronectin. Already after 24 h of culture, proliferation increased in the presence of collagen type IV and laminin, whereas collagen type I caused a moderate decrease of cell growth (Fig. 3E). From 48 h onward, we observed a general decrease of cell proliferation independent from the ECM component (Fig. 3F,G), whereas RSMCs grown on collagen type I and laminin showed a significant increase in cell augmentation compared with cells grown on plastic. Although exposure of RSMCs to collagen type IV and fibronectin resulted in cell division, a lesser but still statistically significant proliferation response was observed (Fig. 3H).

Due to evidence that exogenous ECM components obviously affected the proliferative behavior of RCMs as well as RSMCs, we carried out additional studies to test a possible influence of ECM component combinations. Therefore, we cultured both cell types on so called “double coatings” and analyzed their proliferation rates on days 1, 2, 4, and 8 of culture.

Data obtained within these studies after 8 days of culture showed a definitive and significant influence of ECM combinations on the cellular proliferative behavior (Fig. 4). The results in general demonstrate a detectable influence on cellular proliferation, whereas the effect is more significant for RCMs than for RSMCs. Several coating combinations containing collagen type I resulted in a decrease of RCMs; double coatings with collagen type I caused increased proliferation rates of RSMCs. Collagen type I combined with laminin in particular gave rise to the most significant progression of proliferating RSMCs (Fig. 4B), while collagen type I/collagen type IV caused the most obvious reduction of RCM proliferation rates (Fig. 4A). Laminin and fibronectin coating combinations resulted in more or less moderate changes in proliferation rates for RSMCs (Fig. 4B) compared with uncoated surfaces except for the combination...
collagen type I/laminin—as mentioned before. More significant differences could be shown for RCMS. In this case, combinations with laminin and fibronectin caused significant increasing proliferation rates (Fig. 4A), except for the combination laminin/collagen type I, which caused an explicit decreased proliferation of RCMs. Collagen type IV/collagen type I as well as fibronectin/collagen type I also effected decreasing proliferation rates of RCMs (Fig. 4A).

Finally, we analyzed the data in regard to the order of the tested ECM components used for the double-coating experiments (Fig. 5). After 8 days of culture, collagen type I in general demonstrated the most considerable influence on the proliferation rates of the cultured cells. RCMs reacted to collagen type I with decreasing cell growth (collagen type I/collagen type IV), whereas collagen type I used as second coating substance amplified the decreasing effect manifold (collagen type IV/collagen type I) (Fig. 5A). For RSMCs grown on collagen type I coating combinations, proliferation rates remained more or less unchanged with the exception of collagen type I/laminin which induced a significant increase in cell growth and collagen type IV/collagen type I which

**FIG. 4.** Proliferation rates of RCMs (A) and RSMCs (B) in response to combinations of exogenous ECM components on day 8 of culture (data are mean ± SD; \( P < 0.05 \); \( n = 6 \) for all values; *, significant data). CI, collagen type I; C IV, collagen type IV; LN, laminin; FN, fibronectin.
caused a decrease of proliferation rates (Fig. 5B). Combinations of collagen type IV and laminin showed more or less no effect compared to uncoated surfaces for both RCMs as well as RSMCs. Collagen type IV/fibronectin double coatings and fibronectin/laminin caused a significant increase of RCM cell growth (Fig. 5A), whereas these combinations of ECM components left the proliferation rates of RSMCs more or less unchanged (Fig. 5B). Evaluation and analysis of absolute cell numbers (Tables 1 and 2) proved the stated influence of single coating and coating combinations on the proliferation behavior of RCMs as well as RSMCs.

**DISCUSSION**

Cell transplantation is currently gaining a growing interest as a potential new means of improving the prognosis of patients with cardiac failure (11). The overall objective of cell therapy and tissue engineering in this field is to repopulate injured cardiac tissue with contractile cells to replace dead cardiac myocytes and to restore the functionality of the failing heart. However, first clinical results are controversial and demonstrate the need to better understand cardiomyocyte and skeletal muscle cell biology, and the way to successfully implant new cells in diseased heart tissue.

EMCs provide an adhesion substrate for transplanted cells and can regulate the organization of cells seeded into the matrix and the subsequent proliferation of these cells to form new tissues (11). The majority of previous investigations into mechanisms that regulate proliferation of cardiomyocytes and skeletal muscle cells have been focused on the role of growth factors and their receptors (12). Some studies begun to elucidate the critical role of ECM focused on attachment and survival of cardiac cells (13,14,16). Lundgren and coworkers showed that cardiac myocytes attached equally well to collagen type IV and laminin, but did not attach to collagen type I (13). Bird et al. found out that laminin provided the best overall cellular attachment of cardiac myocytes compared with other reagents examined (e.g., fetal bovine serum, gelatine, poly L-lysine, Matrigel) (14).

As there is little information available about the possible influence of ECM components on cardiomyocyte and skeletal muscle cell proliferation rates, our work focused on this particular question and demonstrated an obvious effect on the proliferative behavior of cardiomyocytes as well as skeletal muscle cells cultured on singular ECM components such as collagen types I and IV, laminin, and fibronectin up to 8 days. The effect differed depending on both cell type and ECM components, whereas collagen type I showed the most impressive influence on cell growth. Proliferation rates of cardiomyocytes decreased significantly in contrast with skeletal muscle cells cultured on singular ECM components such as collagen types I and IV, laminin, and fibronectin up to 8 days. The effect differed depending on both cell type and ECM components, whereas collagen type I showed the most impressive influence on cell growth. Proliferation rates of cardiomyocytes decreased significantly in contrast with skeletal muscle cells, which grew significantly better in the presence of collagen type I.

Additional effects could be pointed out by using combinations of ECM components. Depending on the order of ECM components, the proliferation rates of both cell types could be increased as well as decreased. Several ECM combinations containing collagen type I caused a significant decrease of RCM proliferation rates. This obvious effect could be amplified when collagen type I was used as second component of the tested ECM coating combinations. Proliferation of RSMCs, however, only increased significantly after cultivation on collagen type I and laminin combination. The remaining combinations of ECM components did not cause any significant proliferative effect on RSMCs compared to cells cultured on uncoated surfaces.
The fact that the investigated cell types revealed a quite different proliferative behavior should be kept in mind in connection with the choice of cells used for cardiac tissue engineering and myocardial cell transplantation purposes. Further studies should be carried out concerning the possible role of ECM components in differentiation processes. These investigations will elucidate how to modulate the proliferation and differentiation of cells appropriately in vitro and in vivo by controlling their microenvironment. As the cardiac ECM also plays an essential role in pathophysiologic processes such as cardiac dilatation, hypertrophy, and ischemic injury, and abnormalities in ECM composition and concentration also lead to heart failure (21), results obtained by further studies may give useful insights in the role of ECM during development of cardiac failure.

This knowledge will enable tissue engineering to emerge as the available therapeutic option for the treatment of cardiac diseases.

Acknowledgments: This work was supported by the Ludwig Boltzmann Institute for Cardiovascular Research, Vienna, Austria.

REFERENCES