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Cell Sheet Technology using Human Umbilical Cord Mesenchymal Stem Cells for Myocardial Tissue Engineering

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Abstract

With limited regenerative capacity and most complex structural and electrophysiological properties; recapitulating the cardiac tissue is a challenging task for the researchers. The cell injection was found unreliable due to the cell loss and low retention of the transplanted cells. This could be overcome by the technique of cell sheet engineering. Scaffold free, thick, cell dense, three dimensional constructs could be generated for suture free transplantation. For the generation of the cardiac constructs the neonatal cardiomyocytes and myoblasts were mostly used. They were tedious to isolate and culture and the risk of arrhythmogenic foci prevailed. Hence the concept of differentiating a suitable allogeneic cell source to myocardial lineage seemed relevant. Human umbilical cord mesenchymal stem cells (hUCMSCs) are emerging with the assistance of differentiating agents and cell sheet engineering for addressing the cardiac regeneration. The preliminary report on this regard has been published. The cells attained cardiomyocyte-like morphology with the expression of alpha-actinin and myosin heavy chain on culturing with cardiac conditioned medium and the inducer sphingosine-1-phosphate. It presented cardiomyocyte-like action potential and voltage gated currents. Hence the cell sheet engineering approach with cells differentiated to cardiac lineage using specific agents is a recent area to be explored.

Keywords: Cardiac tissue engineering; Umbilical cord MSC; Cardiac construct; Differentiation; Cell sheet technology

Introduction

The mighty task of more than 100,000 beats per day, sending around 2,000 gallons of blood; the heart works tirelessly to meet our body's demand of blood. Cardiomyocytes (CM), the key cells of the heart; were thought to be terminally differentiated, turning to binuclear or polyploid after final division at birth [1-3]. The response to mitotic signals were thought to be, by cell hypertrophy and not by increase in cell number [4,5]. In a study similar to pulse chase experiment, Bergmann et al. [6] proved that mitotic turnover of cardiomyocytes would be around 1% per year by the age of 25. But again, this further decreases with age, clearly indicating the modest regenerative capacity of heart [7].

Hence, to address this loss of functional CMs due to ageing or due to a major insult to the myocardium, strategies are to be designed such that, either the promotion of cell division of the existing CMs or the stem cells around would address the problem [8]. It may either be by targeting and activation of cell-cycle re-entry pathways or by the dedifferentiation the CMs [9]. The adverse micro-niche created due to the insult, further hampers the extent of cell division and survival. Hence, the major challenge to the endogenous restoration would be the insufficient number of available stem cells. The pharmacological interventions can slow down the progress of damage but, it just mitigates the symptoms for a short span, with no role in repair or regeneration of the heart. Hence the heart needs exogenous support for its regeneration.

Cell Based Therapy

The administration of cells to the infarcted heart had been the mode of therapy regeneration. The possibility of injection of CMs isolated from mouse hearts was first described by Soonpaa et al. [10]. The cell suspension was injected to the damaged site via thoracotomy, into coronary arteries. The studies accounted for only moderate success due to difficulty in controlling the size, shape and target area of the grafted cells. This is very evident, even from the recent studies by Nascimento et al. The human umbilical cord mesenchymal stromal cells did not engraft to the infarcted heart while it could attenuate remodelling by cardioprotective paracrine factors and by endogenous cell-activation mechanisms [11]. Further assays were performed with merely the conditioned media of umbilical stromal cells and proved to induce vasulogenesis in matrigel seeded with human umbilical vein endothelial cells [11]. Thus, high rate of cell wash out resulted in low retention and reduced survival rate in host tissue [12]. The survival of transplanted cells was questioned by low blood flow and oxygen availability in the infarct zone. This further hampered the cardiac regeneration. Thus the need, to obtain a therapeutic biologic implant, that resembles the functional features of cardiac muscle, to restore the cardiac structure and muscle mass, has still remained an unanswered question. To overcome these problems, the tissue engineering approach has become a second-generation cell therapy. Engineering myocardial tissue comprises the fields of cell biology (cell source) and material science (scaffolds/substrates). It aims at regenerating or replacing the injured myocytes and eventually improving cardiac function after myocardial infarction (MI) [9].

Cell Sources for Cardiac Regeneration

An important aspect for cardiac regeneration is the optimal cell source. It should be functionally active, should be easy to harvest, proliferative, non-immunogenic, with structural, electrophysiological and contractile properties similar to that of CM [13]. Foetal cardiomyocytes (FC) were the first to investigate as they possess similar electrophysiological properties to CMs. On cell injection, they showed survival preventing post-infarction heart failure. The alignment with the host cells, with defined cell-cell contacts, makes them the ideal donor cell type [14]. However, they are tedious to harvest, culture and are allogeneic, causing immune response in the host [15]. Hence numerous experiments have been carried out to demonstrate the potential cell types to initiate neo-vascularization and repair/regeneration of the injured myocardium.

Eliminating the arrhythmogenic foci as in FCs, the autologous skeletal myoblasts showed survival with differentiated muscle fibres after transplantation [16]. Also, the resident cardiac stem cells, though sparse in the scene; when locally stimulated by hepatocyte growth factor and insulin-like growth factor-1, it reduced the scar area by half, owing to the degradation of collagen proteins by the matrix metalloproteinase synthesised by them [17]. But these cells have the major disadvantage that, they have to be procured from the same host, which is invasive and risky.

The advances in the embryonic and induced pluripotent stem cell research gave a new pace for the cardiac differentiation studies. They have shown to repeatedly differentiate into functional cardiomyocytes by the addition of defined factors such as activin-A, VEGF-A, BMP-4, bFGF and DKK-1 with the positive expression for cardiac troponin T or β-MHC [18,19]. But ethical issues and teratoma formation are their unavoidable concerns.

Mesenchymal stem cells (MSCs) are extensively researched upon, due to the secretion of paracrine factors which play a major role in the recruitment of adjacent stem cells. Their plasticity and easy detection with surface markers, makes them attractive [20]. Hence their angiogenic and antiapoptotic mechanisms can be used for the repair and regeneration of the heart [18,21]. Induction using various agents like 5-azacytidine,TGF β2 or/along with rat cardiomyocyte extracts, resulted in the expression of cardiac markers like GATA4, Nkx2.5, myosin heavy chain and troponin I at the protein level [22-25].

Human umbilical cord blood-derived mesenchymal stem cells (UCBMSCs) have been recently reported to limit ventricular remodelling by minimizing cell loss when engineered to a construct with fibrin patch. They were modified to co-express luciferase and fluorescent protein reporters for non-invasive bioluminescence imaging in post-infarct mice. The patch was found to adhere to the infarct zone of the heart with functional vasculature with early cell proliferation and differentiation [26]. The ethical issue related to collection of umbilical cord blood can be overcome by the use of umbilical cord Wharton's jelly mesenchymal stem cells (hUCMSCs) which is a biological waste of parturition and can be easily obtained post-partum causing no pain or harm to the mother and baby [20]. They are superior to bone marrow derived MSCs in availability, proliferation, ease of isolation, immuno-modulation with higher frequency of proliferation and colony-forming units (CFU). They can be frozen and stored for long term, than BM-MSCs. They exhibit lower expression of HLA-class I, lack expression of HLA class-II surface markers with lower risk of viral contamination [25,27] and lesser complications of ethical clearance [28].

Thus, hUCMSCs, are emerging cell source for cardiac tissue engineering. They can be differentiated to the cardiac lineage using various chemical or biological agents like vascular endothelial growth factor (VEGF), zebularine [29], 5-azacytidine [22,29], ghrelin hormone [30], sphingosine-1-phosphate [31], low levels of dexamethasone [32] and mechanical or electrical stimulation [33]. The increased expression of the genes related to matrix remodelling by the hUCMSCs is more favorable for their differentiation [34]. Hence they are found to be copious and cost effective cell source for tissue engineering applications [35]. However, the studies have been limited to *in vitro* culture and its differentiation. Also, the functional characteristics of cardiomyocytes, differentiated from hUMSCs have not been fully elucidated till date [31]. Thus, the choice of most appropriate cell source for myocardial tissue engineering still remains an important topic of research.

Methods of Cell Sheet Engineering

Cell sheet engineering (CSE) advocates the "scaffold free" and "suture free" technique of tissue engineering. The technique helps in the spontaneous detachment of the cells as continuous sheets negating the use of denaturing enzymes like trypsin or dispase and mechanical disruption. The cell-cell junctions, cell surface proteins as well as ECM are preserved in cell sheets. They can adhere tightly onto the host tissues without suture or cell loss after transplantation [22,36]. Cell sheet transplantation shows greater cell survival than dissociated cell injection in *in vivo* studies [12]. The latest application of it, being the generation of thick three-dimensional cell-dense tissue construct by the sequential stacking of confluent cell sheets, which may be even patterned [37].

There are several methods for the generation of cell sheets as per the responses to temperature [37-40], pH [41], ionic strength [42], electrochemical desorption [43] and light [44]. The thermo-responsive polymers are the widely used. This could be, by the use of poly (N-isopropyl acrylamide) [37], N-isoproplyacrylamide-co-glycidylmethacrylate (NGMA) [39,40] or methyl cellulose [5]. Kaneko et al. were the pioneers in the study of temperature-sensitive culture substrates [38]. On lowering temperature, the smart surface changes from hydrophobic to hydrophilic, making it non-cell adhesive. Rapid hydration and extension of polymer chains helps in the detachment and retrieval of intact cell sheets [36].

The corneal sheets were generated to address the issues of graft rejection and donor shortage. We have previously reported the production and evaluation of bioengineered corneal epithelium from limbal tissue [39] and the generation of a layered construct from rabbit corneal endothelial cells, stromal fibroblasts and epithelial cells [40] using in-house developed NGMA. As an alternative to corneal epithelial cell sheets, the potential of autologous oral mucosal epithelial cell sheets were also reported [46]. The application of CSE has been efficiently used for the generation of periodontal ligament cell sheets, urothelial cell sheets, co-cultured cell sheet of hepatocytes and endothelial cells using patterned dual thermo-responsive surfaces (cross reference [37]).

A simple method of cell sheet retrieval is by coating fibrinogen monomers mixed with thrombin on the culture surface. Intact cell sheets of neonatal rat cardiomyocytes were obtained. This is due to the digestion of the fibrin by the intrinsic protease [47]. This may be applicable for other cell types also.

Another technique of cell sheet engineering is by the induction of cells by Vitamin C. The BMSC, UCMSC, periodontal ligament cells were induced with 20 µg/mL of Vitamin C until the edges of the confluent monolayer wrapped. The sheet was detached smoothly using a crooked syringe needle. The induction, resulted in increased telomerase activity causing the upregulation of fibronectin, integrin β1and extracellular matrix type I collagen. This could be defined as a new, easy and cost effective method for cell sheet retrieval [48].

Development of tissue construct: Many protocols have been reported for the effective stacking of cells sheets to create a 3D construct. The initial method advocated was by pipetting with broad/cut tips [49]. The sheets could be placed one above the other but with utmost care without tampering the sheets. The use of the hydrogel-coated, plunger-like manipulator designed by the Okano group; with the combination of low-temperature (20°C) treatment; intact cell sheets of neonatal rat cardiac cells were stacked without cell damage or shrinkage [49]. As extension to the use of the manipulator, they designed an automated cell sheet stacking apparatus, to fabricate 3D constructs. A five layer human skeletal myoblast construct (70-80 µm) was developed within 100 min [50]. The centrifugation methods were reported to further decrease the duration for cell attachment. C2C12 mouse myoblast sheet were found to attach to the culture surface within 3 min after 3 min centrifugation (12-34 x g) rather than 20 min for the control. This resulted in decrease in the manipulation time by two-thirds [51].

Gel casting method using gelatin was reported as a versatile platform for the fabrication of scaffold-free 3D tissues. The gelatin solidifies on lowering the temperature and holds the retrieved cell sheet onto it. On bringing back to 37°C, it melts and can be removed. The sheets could be aligned with required orientation without losing anisotropy [52].

These varying techniques of cell sheet engineering improved the possibility of heart regeneration using cell sheet constructs and opened up new doors for research in cardiac tissue engineering. The cells could remain in close proximity and, in synchrony, with the electrophysiological environment of the myocardium allowing suture free epicardial transplantation [53]. Sekine et al. have found improved survival of cardiac cell sheet transplantation to direct cell injection in rat MI model [22]. The cell survival was more than ten times at four weeks after transplantation [12]. Transplanted cell sheet grafts prove long-term survival while retaining the original functions and growth in accordance with the host growth. This could be even more practical for the paediatric patients in the clinical point of view.

Cell Sheet Engineering for Cardiac Regeneration

The need of the hour is to define an efficient, economic and reliable method of using the ideal cell type for improved cell delivery and cell retention at the infarct zone amidst the adverse micro-niche and enhance their survival post-transplantation. This can be overcome by the following approaches like injecting the cells with bioactive *in situ* polymerizable hydrogels, preconditioning with pro-survival agents, genetic manipulation to limit cell death or via the transplantation of the tissue-engineered patch (cross reference [17]); of which the last technique support survival of delivered cells for long-term and proved to reconstruct the cardiac tissue both structurally and functionally [12].

Rat neonatal cardiomyocytes seeded on the mix of fibrinogen monomers with thrombin, enabled the generation of triple-layered well-organised construct with connexin 43 expressions [47]. Similarly, myoblast sheets implanted to infarcted Lewis rats resulted in improved cardiac function with more elastic fibers and adequate blood supply. The reduction in fibrosis was found at the infarct zone [54].

The major limitations of cardiac constructs are the poor vascularization limiting the viable size of constructs. Three neonatal rat cardiomyocytes sheets were pipetted and stacked one above the other as a construct. Ten such constructs, each was transplanted to subcutaneous implant site of F344 nude rats at an interval of 1 day by polysurgery. This facilitated the fabrication of around 800 µm thick, cell dense, viable construct with vascularity, eliminating mass transport limitations [55]. Neonatal cardiomyocytes have been mostly used in the studies for cardiac regeneration. But the difficulty in isolating and culturing them, limits their use. Also, when highly enriched CMs (CM>90%) were plated on thermo-responsive surface, failure of cell sheet formation was reported, while CMs along with vascular cells gave better results [56].

Hence, the best solution is to differentiate an allogeneic stem cell into myocardial lineage. The rat bone marrow MSCs sheets differentiated using 5-azacytidine, grown on thermo-responsive methylcellulose surface were inserted into sliced porous acellular bovine pericardia and transplanted to rat MI models. They could improve the cardiac function with viable cells adhered to the fibronectin mesh of the scaffold [22]. Immunogenicity, age and pathological conditions of donor would vary in the bovine extracellular matrix [57].

Human induced pluripotent cells (hiPS) were used by Kawamura et al. to study the effect on cardiac regeneration. It was generated from human dermal fibroblasts by transfection of Oct3/4, Sox2, Klf4 and c-Myc. The induction by WNT signaling molecules yielded 90% of differentiated cells with α-actinin, Nkx2.5 and cardiac troponin T expression. The cell sheets were transplanted on to myocardial infarcts in an ischemic cardiomyopathy porcine model and showed improvement in cardiac function. This culture system could be the basis for clinical use of hiPS cells in cardiac regeneration therapy [58]. But the risk of teratoma formation remains a major concern [19]. Also, evidence of any electrical integration between the grafted and host tissues could not be identified as the origin of the cells varies considerably [58].

The intact sheets of hUCMSC which has been differentiated to cardiac lineage using sphingosine-1-phosphate, has been reported by Zhao et al. They exhibited a cardiomyocyte-like morphology and the expression of alpha-actinin and myosin heavy chain on culturing with cardiac conditioned medium and sphingosine-1-phosphate. It presented cardiomyocyte-like action potential and voltage gated currents [31]. The potential of hUCMSCs to yield potent cardiac constructs are yet to be explored. We have retrieved a differentiated cardiac cell sheet from hUCMSC by the single dose treatment of 5-azacytidine and have shown the presence of cardiac markers after differentiation (under communication). The cardiac construct thus obtained could be easily transplanted to MI models with a suture free procedure, thereby increasing the therapeutic area of interest [54]. Kadivar et al. had stated human hUCMSCs, to be a better source of seed cells for generating cardiomyocytes [59]. However, the scope of using hUCMSC for cardiac tissue engineering using cell sheet technology still remains to be exploited.

Conclusion

A tissue engineered myocardial construct using allogeneic cells like differentiated hUCMSCs to cardiac lineage, with native extra cellular matrix without an externally provided scaffold would be a better advancement in treating myocardial infarction (MI). But the functional characteristics of cardiomyocytes differentiated from hUMSCs have not been fully elucidated till date.

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