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BUILDING A TISSUE IN VITRO FROM THE BOTTOM UP: IMPLICATIONS IN REGENERATIVE MEDICINE

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Abstract

Tissue engineering aims at creating biological tissues to improve or restore the function of diseased or damaged tissues. To enhance the performance of engineered tissues, it is required to recapitulate in vitro not only the composition but also the structural organization of native tissues. To this end, tissue engineering techniques are beginning to focus on generating micron-sized tissue modules having specific microarchitectural features that can be used alone as living filler in the damaged areas or serve as building blocks to engineer large biological tissues by a bottom-up approach. This work discusses the shortcomings related to traditional "top-down" strategies and the promises of emerging ''bottom-up" approaches in creating engineered biological tissues. We first present an overview of the current tissue-building techniques and their applications, with an analysis of the potentiality and shortcomings of different approaches. We then propose and discuss a novel method for the biofabrication of connective-like micro tissues and how this technique can be translated to cardiac muscle fabrication.

Introduction

There is an increasing demand in regenerative medicine to repair and restore the function of injured, degenerated, or congenitally defected tissues. In a wide range of pathology, neither native nor purely artificial implantable materials can adequately replace or repair these damaged tissues. The capability of a tissue to self-repair is limited to the case of bone[1] or skin,[2, 3] provided that the damage is not invasive such as a small injury or a superficial wound. In many tissues such as myocardium[4] and cartilage,[5] or in the case of large bone defect and deep skin wound, the self-repairing capability is lost and surgery becomes necessary. To overcome such limitations, tissue engineering focuses on the in vitro fabrication of living and functional tissue that can be implanted in the damaged zone to restore the healthy status. The classical tissue engineering approach (herein referred to as "top-down") is based on the concept of seeding cells into preformed, porous, and biodegradable polymeric scaffolds that act as a temporary template for new tissue growth and reorganization. Such cellular construct is then processed in bioreactors that provide a viable molecule microenvironment and simulate physiological conditions that furnish suitable stimuli for cell survival, differentiation, and extracellular matrix (ECM) synthesis.[6] The main drawbacks of this approach are related to: (1) the difficulty in reproducing adequate microenvironmental conditions in a three-dimensional (3D) thick structure at the pericellular level; (2) recreating the architecture of native tissue; (3) problems in selecting the ideal biomaterial scaffold for a given cell type; (4) time constraints in achieving a high enough cell density and the homogeneous cell distribution necessary to construct a viable tissue. By studying the nature of living tissues, it is possible to observe that most of them are composed of repeating units on the scale of hundreds of microns, with well-defined 3D microarchitectures and tissue-specific functional properties. The recreation of these structural features is becoming significant in enabling the resulting tissue function in vitro.[7] In light of this observation and to overcome the limitation of top-down tissue engineering, recent efforts have been devoted to bottom-up[8-16] approaches aimed at generating a larger tissue construct by assembling smaller building blocks that mimic the in vivo tissue structure of repeating functional units. These building blocks can be created in a number of ways, such as self-assembled cell aggregates,[17-18] microfabrication of cell-laden microgel,[7] creation of cell sheet,[9] and microfabrication of cell seeded microbeads.[19-20] Once obtained, these building blocks can be assembled in larger tissue through a number of methods including random packing, stacking of layers, or direct assembly. A bottom-up approach has been used by Du et al.[7] to direct the assembly of cell-laden microgels to generate 3-D tissue with tunable microarchitecture and complexity. In a different way, McGuigan and Sefton[8] proposed an approach in which rod-shaped collagen microgels, seeded with HepG2 hepatocytes on the inside and endothelial cells on the surface, were "packed" together within a bioreactor and perfused with medium; this demonstrated the possibility of using micron-sized modular components in a biomimetic fashion to assemble uniform, potentially scalable, vascularized tissue-engineered constructs containing multiple cell types. Even in the field of cardiac regenerative medicine, some groups have taken advantage of modular tissue engineering in creating a cardiac tissue construct. For example, beating cardiac sheets have been generated by stacking sheets of cells for patches obtained by means of "cell sheet engineering."[9] In this technology, a cell sheet produced by a 2D cell culture system works as a building block for organ-like structures. By using thermo-responsive culture dishes, cell sheets are easily harvested as intact monolayers of about 30 µm in thickness that can be layered on top of one

another to create a 3D construct, such as thick cardiac muscle.[21-24] The advantage of cell sheets is that an entirely natural neotissue assembled from cells with a mature ECM can be engineered. Nevertheless, this technology has a number of shortcomings, such as handling difficulties with the cell sheets and the limited number of sheets that can be effectively layered without core ischemia or hypoxia.[9, 25, 26] However, many of the studies dealing with the fabrication of tissue-equivalent rich in endogenous ECM have neglected the organization and assembly of de novo synthesized ECM.[26-28] In fact, the resulting tissue-equivalents are generally composed of cell aggregates with spare ECM molecules far off from a correct and mature tissue organization.

To address this issue, we propose a bottom-up method to fabricate micron-sized tissue of connective origin by coupling cells and micro-scaffold. Despite other bottom-up strategies that are completely scaffold-free, we use a porous micron-sized scaffold as a temporary support that plays a crucial role in guiding the correct spatial organization of the de novo synthesized ECM. This way, the microtissue is more than a mere cell aggregate: it represents a more complex living structure in which the cell works as tissue builder. These microtissues are then used as building blocks to create a 3D dermal equivalent. Finally, we show how this method can be translated to cardiac-muscle fabrication.

Fabrication of Dermis Equivalent In Vitro

Tissue Modules Realization

Microtissue modules have been obtained by means of dynamic cell seeding of fibroblasts on porous gelatine microcarriers using a spinner flask bioreactor as depicted in Step 1 of Figure 1A. Several studies demonstrated that this particle cultivation technique is more effective than cell culture on flat substrates such as culture dishes.[29, 30] We report that under optimal culture conditions, cells were able to adhere, proliferate, and, in particular, synthesize ECM components to form a thin layer of de novo-produced tissue around the microbeads. This micrometric tissue wrapped around and within the porosity of the scaffold constitutes micrometric tissue precursors (µTPs) for further large-tissue construction.[19, 20] The process for µTP formation consisted of a dynamic cell seeding performed in a spinner flask bioreactor, which was loaded with cells and porous gelatine microbeads in the ratio of 50 cells per microbead and operated at 30 rpm. The disappearance of free cells from the inoculated spinner cultures was considered to indicate the attachment of cells to the microcarriers. By determining the concentration of the fibroblasts in the culture medium during the first hours, it was possible to observe that the cell density in the culture medium (cells ml^{-1}) decreased by up to 60% after 6 hours because of cell attachment to the microbeads and cell death. The number of cells per microbead increased during culture time (from 20 to 80), indicating cell attachment and proliferation. The histological images reported in Figure 1B show that cells were able to synthesize ECM components, which act as biological glue generating aggregates of µTPs (Figure 1C). The spontaneous assembly of µTPs after a few days in spinner culture suggested that this phenomenon could be exploited to induce their assembly in a 3D tissue construct of the desired shape by means of the second step of the process-maturation phase (Figure 1D).

From Micro- to Macrotissue

Due to their self-assembling capability, µTPs have been considered an ideal "material" for biofabrication of 3D tissue constructs. They can be assembled in an appropriate assembling chamber, and the tissue layers surrounding them allow their fusion through cell-cell and cell-matrix interactions. Following this strategy, a 3D functional dermal tissue equivalent has been created (Figure 1D, E),[20] and an assembling chamber able to

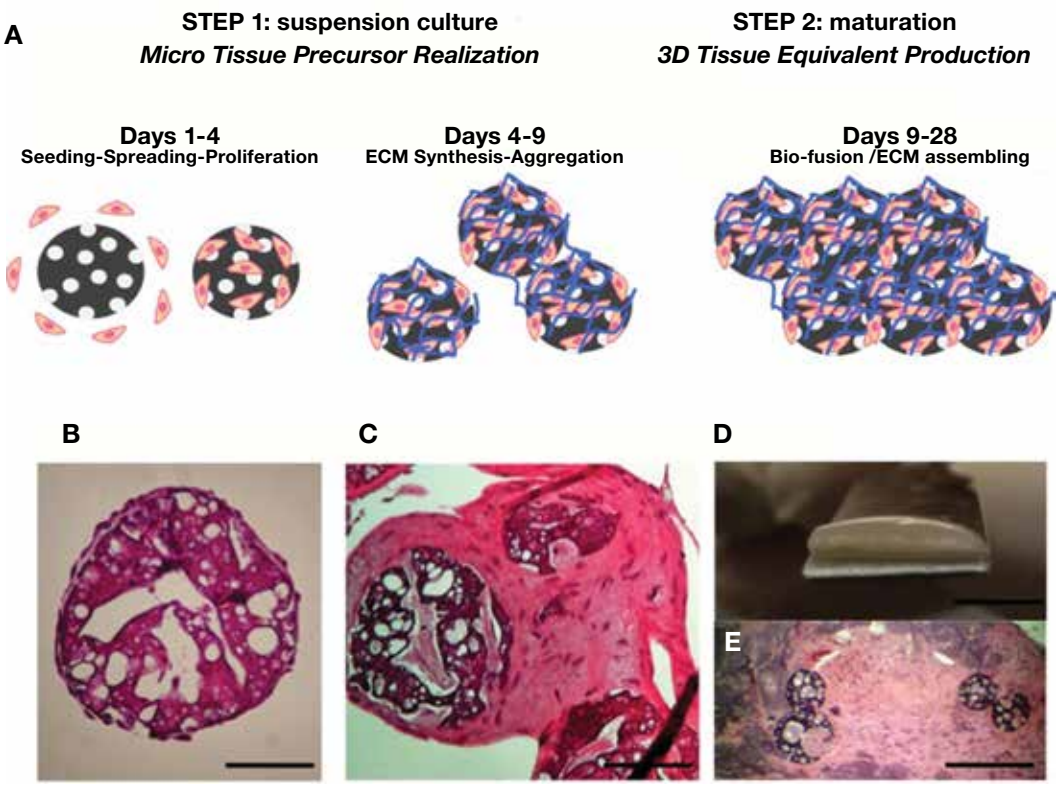

Figure 1. Description of 2 STEP process used to generate 3D tissues in vitro. First row: cell seeding and micro-scaffold colonization at an early time; ECM synthesis and formation of small aggregates named µTP; (A) fusion and assembly of µTP to produce large 3D tissues. Second row: (B) histology of µTP during first days of spinner culture; (C) histology of small aggregates of µTP: ECM is well visible and stained in pink, cells are stained in dark pink and microscaffold in purple; (D) 3D tissue obtained by bio-fusion of µTPs; (E) histology of cross section of the 3D tissue. ECM: extracellular matrix; µTP: micrometric tissue precursors.

work under both static or perfusion conditions has been designed (Figure 2A, B). It was observed that after 1 week in the maturation chamber under static conditions, the building blocks were able to assemble, leading to a compact tissue equivalent. Histological images show that abundant ECM that connects µTPs and organized collagen fibers were present in the matrix (Figure 1E). By inducing µTP assembly and 3D tissue equivalent maturation under different hydrodynamic culture conditions, we have been able to assess the strong effect of culture conditions on the assembly of neosynthesised tissue and its mechanical properties (Figure 2). It is well known that bioreactors operating under perfusion flow ensure an efficient nutrient transport and avoid necrotic region formation in the center of a 3D tissue equivalent. However, continuous perfusion can induce a washing out effect of the neotissue component. This hypothesis has been confirmed in our experiments by comparing the collagen and glycosaminoglycans (GAG) content in the culture medium performed under continuous perfusion flow (CF) with the content in the culture medium performed under mixed flow (MF, 1-day perfusion and 1-day tangential, Figure 2A, B). According to the biochemical results, we found that the mechanical properties of the tissue equivalent were affected by culture conditions. After 8 weeks of culture, the shear elastic modulus (G') of the tissue equivalent kept under CF did not show any significant variation (from 2.9 x 10^3 Pa to 3.2 x 10^3 Pa). Conversely, under MF conditions the G' modulus almost doubled in 8 weeks, jumping from 2.8 x 10^3 Pa to 5 x 10^3 Pa. In Figure 2B, the shear elastic modulus at a frequency of 1 Hz has been reported for a biohybrid cultured under MF and CF and compared to that of native dermis. The modulus of the sample cultured under MF is remarkably close to that of native dermis, indicating that a firmer structure has been produced under these processing conditions. Moreover, by comparing the results concerning the biochemical composition and the mechanical properties, it is evident that the higher the accumulation of GAG and collagen content in the medium, the lower the modulus of the corresponding biohybrid. Taken together, our results demonstrate the great potential of µTP as functional building blocks in bottom-up tissue engineering. We hypothesized that the added value of using µTP to build up 3D tissue is mainly related to the presence of cells embedded in their own ECM, meaning that cells undergo µTP assembly with low metabolic output. (or: "meaning that cells require low metabolic output to undergo µTP assembly")

Fabrication of Cardiac Muscle Equivalent In Vitro

Cardiac tissue engineering aims to create functional tissue constructs that can serve to re-establish the structure and function of injured myocardium or that can represent in vitro models to study cardiac development and disease. The bottom-up approach previously described to create connective-like tissue in vitro has been used to generate cardiac muscle equivalent (CME). To this end, native cardiac cell population (NCP) seeded into porous gelatine microbeads was used. The NCP was extracted from neonatal Wistar rats according to the protocol described by Naito et al.[28] Once obtained, NCP has been inoculated as full cell population in the spinner flask bioreactor together with porous gelatine microbeads at the ratio of 1,000 cells per bead. Compared with previously described spinner culture conditions used to generate connective-like tissue, the ratio between cell and beads was higher in the cardiac cell culture due to the NCP's low capability to proliferate in vitro. The seeding parameters were slightly different because of the difference in the cell adhesion time between dermal fibroblasts and NCP. While dermal fibroblasts adhere within 4 hours, NCP took at least 3 days to adhere and to spread on the substrate. For this reason, the spinner culture was carried by using a cycle of 10 minutes at rest and 40 minutes at 30 rpm for up to 3 days of culture. After this time, the dynamic culture switched to continuous stirring at the velocity of 30 rpm up to 9 days of culture. During the culture time, NCP was able to

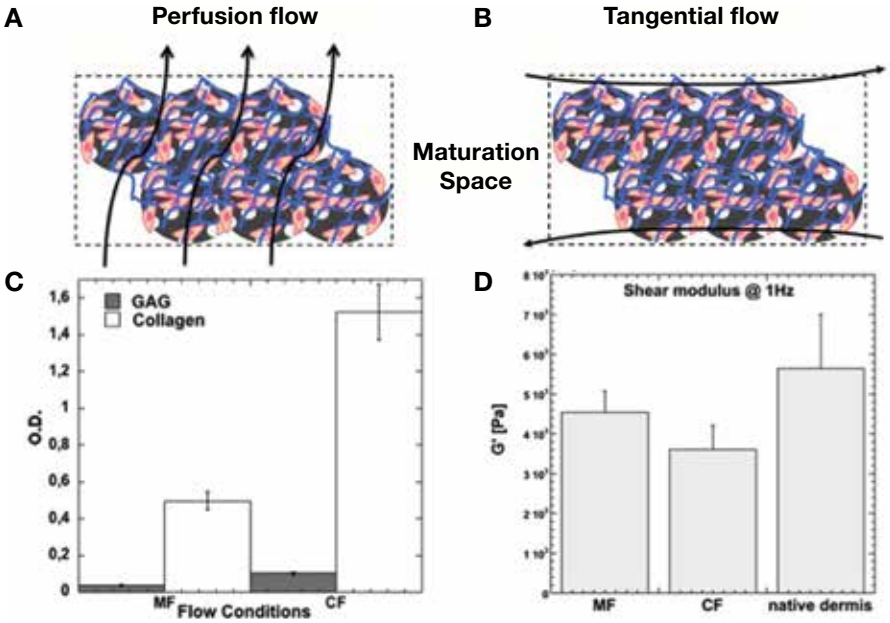

Figure 2. Schematic drawing of the culture condition used to culture the µTPs: (A) direct continuous perfusion, CF; (B) tangential nutrient; mixed flow (MF) consists in alternating 1 day perfusion and 1 day tangential flow; (C) GAG and collagen accumulation in the culture medium at different flow condition; (D) shear elastic modulus of the tissue at different flow conditions. µTP: micrometric tissue precursors; GAG: glycosaminoglycans; CF: continuous flow; MF: mixed flow.extracellular matrix; µTP: micrometric tissue precursors.

adhere to and colonize the microbeads, recreating a structure quite similar to that of native muscle, composed by cardiomyocytes and fibroblast embedded in a collagen network as shown in Figure 3A. CME showed uniform cell distribution and important biological structure such as sarcomere actin filaments; connexin-43 junctions were also present. One of the most important features of CME is the self-beating capability generated by the spontaneous beat of the cardiomyocytes fraction present in the tissue equivalent. After 4 days of culture, cardiac microtissues were collected from the spinner culture, and their spontaneous contraction was monitored by video microscopy. The tissue organization continued till the end of the culture, and at each time point, beating and contracting microcardiac muscle was observed with a beating rate of 45 bpm (Figure 3B). Interestingly, when placed in close proximity, two micro CMEs having different beating frequencies were able to synchronize after 40 minutes. After 1 day, the CMEs underwent a fusion process, and the resulting tissue was made by a microtissue with a single beating frequency.

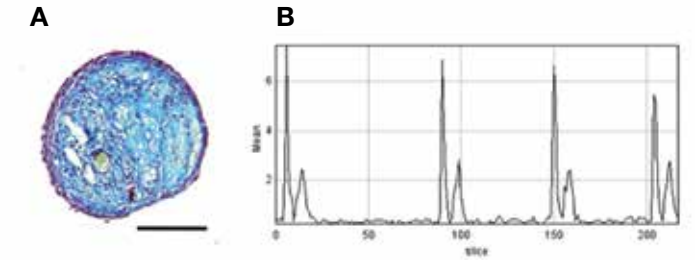

Figure 3. (A) Masson trichrome of micro CME; (B) beating properties of the micro CME; the peak represents a single beat.

The translation of a microscaffold-based bottom-up strategy from dermis to cardiac muscle tissue fabrication resulted in a promising strategy for heart tissue engineering. Despite other techniques such as cell sheet or cellular spheroids, the presence of the microscaffold aids tissue reorganization and ECM synthesis, simultaneously representing a structure that guarantees mechanical support. The presence of the scaffold does not hinder the electromechanical properties of the CME, which showed spontaneous beating and synchronization properties. This indicates that the CME can be used as an implantable living microtissue for infracted zone regeneration.

Conclusion

The strategy presented in this work highlights the production of functional tissue in vitro, made up of endogenous ECM and tunable in size and shape. In light of this, the tissues created could be useful in regenerative medicine as they could be injected as living microtissues in damaged sites or, if assembled in large 3D tissues, could be used as a patch for extended damages. The versatility of this technology paves the way for in vitro biofabrication of several kinds of tissues, leading to an increased availability of "living" tissue or organ substitutes.

Conflict of Interest Disclosure: The author has completed and submitted the *Methodist DeBakey Cardiovascular Journal* Conflict of Interest Statement and none were reported.

Funding/Support: The author has no funding disclosures.

Keywords: tissue engineering, dermis, equivalent, cardiac muscle, bottom-up strategy, regenerative medicine, bioreactor

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