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Procedia Engineering 59 (2013) 138 – 143

Procedia Engineering

www.elsevier.com/locate/procedia

3rd International Conference on Tissue Engineering, ICTE2013

The need for thorough in vitro testing of biomaterial scaffolds: two case studies

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Abstract

Tissue engineered scaffolds are an integral part of future regenerative efforts, whether as carriers for stem cells and growth factors, or as acellular supports on their own. These scaffolds will be inserted at the site of injury, aim to provide temporary mechanical support and promote appropriate cell attachment and growth. Millions of dollars are spent on the development of biomaterial scaffolds, with many put into animal studies and clinical use without proper evaluation, often leading to unfavourable outcomes and associated patient morbidity.

We exposed two novel scaffolds designed for use in tendon regeneration to a series of in vitro tests, the first a decellularised extracellular matrix (ECM), the second a silk-like material. SEM images were taken to evaluate scaffold nanostructure. Primary human dendritic cells were exposed to scaffolds, with FACS analysis of cell-surface activation markers determining scaffold immunogenicity. Growth of primary tenocytes was analysed using live-dead staining and alamarBlue® fluorescence, for cytocompatability. While, phenotypic retention was assessed through real-time PCR analysis of tenocytic genes.

The decellularised ECM demonstrated low activation in the dendritic cell assay, suggesting low immunogenicity. The cytocompatability assays and real-time PCR analyses suggested that the material was indeed suitable for cell growth and differentiation. However, during the third biological repeat, no cells grew on the material, nor did they on the fourth and fifth repeat. In retrospect it appears that following the second repeat a new batch was tested, with very different properties to the first, which accounted for the reduced cytocompatability.

The silk-like material produced some of the most repeatable and promising results with the cytocompatability and real-time PCR analyses, while the SEM demonstrated intertwined fibres suitable for cell alignment. However, the immunogenicity assay

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Selection and peer-review under responsibility of the Centre for Rapid and Sustainable Product Development, Polytechnic Institute of Leiria, Centro Empresarial da Marinha Grande
doi:10.1016/j.proeng.2013.05.103

demonstrated that this material invoked high activation of the primary human dendritic cells, suggesting the material would be unsuitable for in vivo implantation.
These studies have highlighted that without stringent testing in vitro, great time and expense would have been spent taking these materials forward for animal studies and potentially clinical studies, when clearly they are not suitable for these in their current incarnation.

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Keywords: Biomaterial implants; musculoskeletal disorders; tissue engineering; tendon; evaluation.

1. Introduction

Acute tendon injuries are a common clinical problem, occurring in otherwise healthy, active people with an incidence rate of approximately 1:2000 [1, 2]. Damaged tendons heal very slowly and the formation of disorganised fibrotic scar tissue at the wound site alters the biomechanical properties of the tendon, leading to high rates of post-surgical repair failure and re-tearing [1-3]. This is especially true of rotator cuff repairs, where up to 75,000 are performed annually in the US alone [4], with rates of re-tear up to 70% [5, 6].

Tissue engineering aims to augment current surgical procedures to promote full tendon regeneration and recovery. This interdisciplinary approach involves the use of biomaterials to act as scaffolds, placed at the site of injury, to offer temporary mechanical support and ultimately enhance cell attachment, growth and subsequent tissue regeneration [1-3, 7]. Currently there are thirteen FDA approved scaffold devices for use in rotator cuff repair, nine extracellular matrices, three synthetic materials and one hybrid scaffold [8]. Of these devices, only five have published pre-clinical, large animal studies and six have published clinical/surgical follow up studies; and while some scaffolds, such as Wright Medical's GraftJacket, appear to improve clinical outcomes, there is often a lack of control comparisons [9-11]. The general consensus suggests that current available devices, while promising, fail to meet clinical needs [8].

A major problem is that government led funding accounts for 10% of regenerative medicine research, with the rest being made up by venture-based companies. This has led to peer reviewers being replaced by financial analysts and business investors in deciding which science and technology is funded [12]. Which has, in turn, naturally led to greater emphasis on applied research and advanced development, and less on basic science, often resulting in poor fundamental understanding of the interactions between tissue and materials, and often a failure in the product once within clinical use [13].

Indeed, for non-human derived scaffolds to be cleared for use in rotator cuff repairs they must pass through the FDA 510(k) regulatory processes. This generally requires the devices to be equivalent in performance, biocompatibility, safety, stability and sterility to previously approved devices [14]. The majority of tests carried out on novel scaffold devices follow the International Organisation of Standardisation (ISO) protocols for the Biological Evaluation of Medical Devices [15]. Within this protocol there is only one in vitro test, which is for cytotoxicity. This involves either direct culture of cells on the scaffold or a method of extracting leachable materials from the material, exposing them to cells, followed by either quantitative or qualitative analysis of cell viability [15]. This protocol gives a great degree of freedom to researchers, since extraction conditions, cell types and regimens can largely be selected on the basis of what provides the most encouraging results, meaning exact determination of material cytotoxicity will never be accurately known [16].

To date there are several biomaterial scaffolds available to orthopaedic surgeons for treating musculoskeletal disorders, such as rotator cuff repairs, with each scaffold's unique physical, chemical and biological characteristics playing critical roles in their healing effectiveness [8]. Therefore, in order to fully evaluate biomaterial scaffolds, we have developed a succinct in vitro evaluation package. Briefly:

i) Interactions between host cells and the scaffold are often regulated not just by the material itself, but by the specific scaffold topography. Therefore, scanning electron microscopy (SEM) of the biomaterial scaffold will be studied.

ii) One of the key properties of novel scaffolds will be their ability to support host cell growth [2]. To determine if a specific scaffold structure enhances cell grow, qualitative cytocompatability is performed through live/dead staining of primary rat tenocytes (host cells of the tendon) seeded onto scaffolds for 7 days;

iii) Also, quantitative cytocompatability is assessed through alamarBlue® fluorescent analysis of primary rat tenocyte growth on scaffolds over a 14 day period;

iv) Successful tissue healing depends on scaffolds being inductive for host cells to differentiate and synthesise new matrix. Host cell differentiation on these novel scaffolds is measured using gene expression analysis of key markers, assessed through real-time PCR. Expression of other cell differentiation markers is also determined to ensure host cells have not de-differentiated;

v) Finally, successful in vivo implantation of biomaterial scaffolds requires the biomaterial to be immunologically inert and thus lack a major host immune response. Therefore, the immunogenicity of the scaffolds will be assessed in primary human monocyte-derived dendritic cell assays to determine dendritic cell activation and subsequent cytokine release, using flow cytometry.

During each stage of the evaluation, 3D collagen gels derived from rat tail collagen and FiberWire® suture material will be used as control materials, being the closest environment to that of primary tenocytes in vivo (3D gel) and the current gold standard for rotator cuff repair procedures (FiberWire®).

Here we present two case studies of potential new scaffolds for tendon regeneration. The first a decellularised extracellular matrix (Material 1) and the second, a silk-based biomaterial (Material 2), where standard evaluation techniques would have seen these two scaffolds pass through to preclinical studies, likely at a great expense to the companies/researchers involved.

2. Results

2.1. Material 1

When evaluating material 1, SEM analysis demonstrated that the 3D basement membrane of the naturally derived extracellular matrix was still largely intact (results not shown), providing an ideal surface for primary cell culture. Indeed, during the first two biological experiments which quantitatively and qualitatively assessed the material cytocompatability with primary rat tenocytes, it appeared as though the material would prove to be suitable for future study as a biomaterial scaffold in tendon regeneration. However, during the final biological repeat, using a newer batch of material, the tenocytes would not adhere to, or proliferate on, material 1. This was repeated with the new batch of material, to no avail. The difference in material cytocompatability, across biological repeats, is shown in Figure 1.

2.2. Material 2

Material 2 demonstrated high cytocompatability for primary rat tenocytes, both qualitatively and quantitatively, during all biological repeats of the evaluation process (results not shown). Furthermore, analysis of tenocytic gene marker expression suggested that these cells were retaining their tenocytic properties during the two week culture period. Expression of chondrocytic and osteogenic genes was negligible in cells cultured on this material. In comparison, cells cultured within the collagen gels expressed both aggrecan and osterix (SP7) to high levels by day 14 (results not shown).

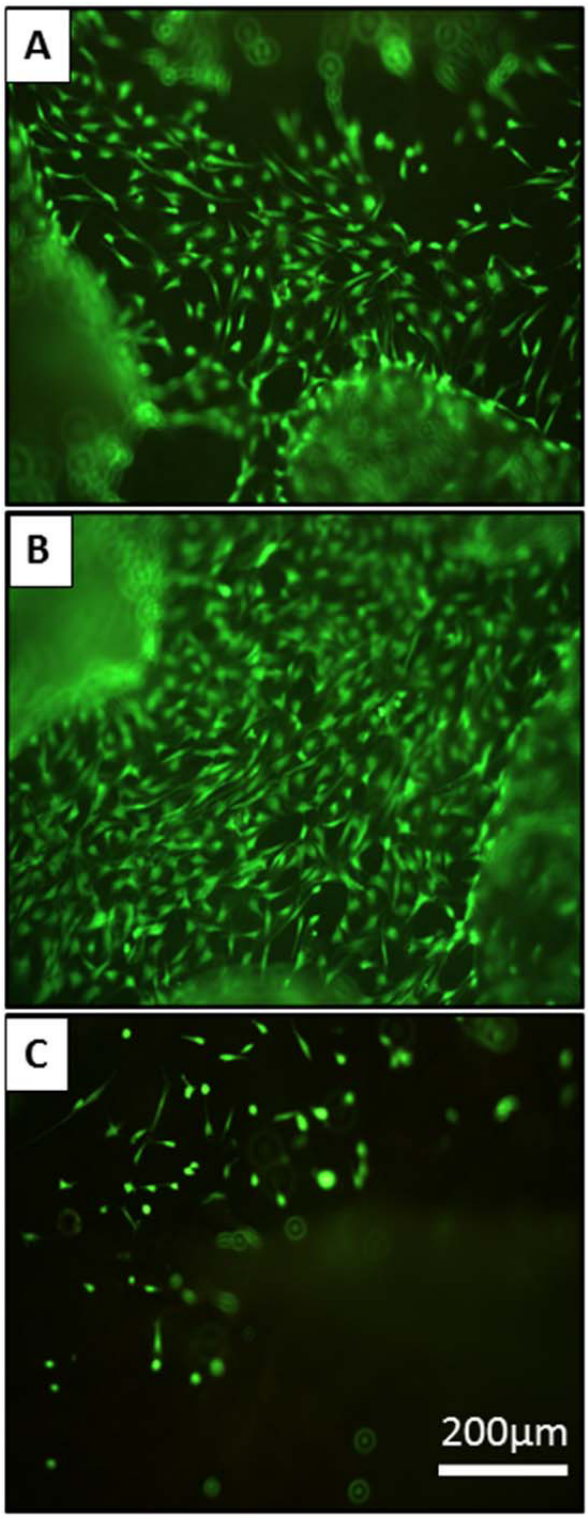

Figure. 1. Calcein-AM “live” staining of primary rat tenocytes cultured for 7 days on Material 1. A: First biological repeat; B: second biological repeat; C: third biological repeat. Biological repeats A and B used material from one manufacturing batches, while C was from a new manufacturing batch.

Following primary human dendritic cell exposure to material 2, the number of cells expressing the immune cell-surface activation markers CD80, CD83 and CD86 was markedly higher compared to untreated cells (Figure 2C). Expression of these markers in dendritic cells exposed to control collagen gels was largely unchanged, while there was a slight increase in the number of cells expressing CD83 and CD86 in cells exposed to the suture material, FiberWire® (Figure 2A and B). Subsequent analysis of the cytokines released from the activated dendritic cells were that levels of IL-1β, IL-6, IL-10 and TNFα were all markedly higher in cells exposed to material 2, compared to untreated cells, though not to the same degree as those exposed to the positive control, LPS (results not shown).

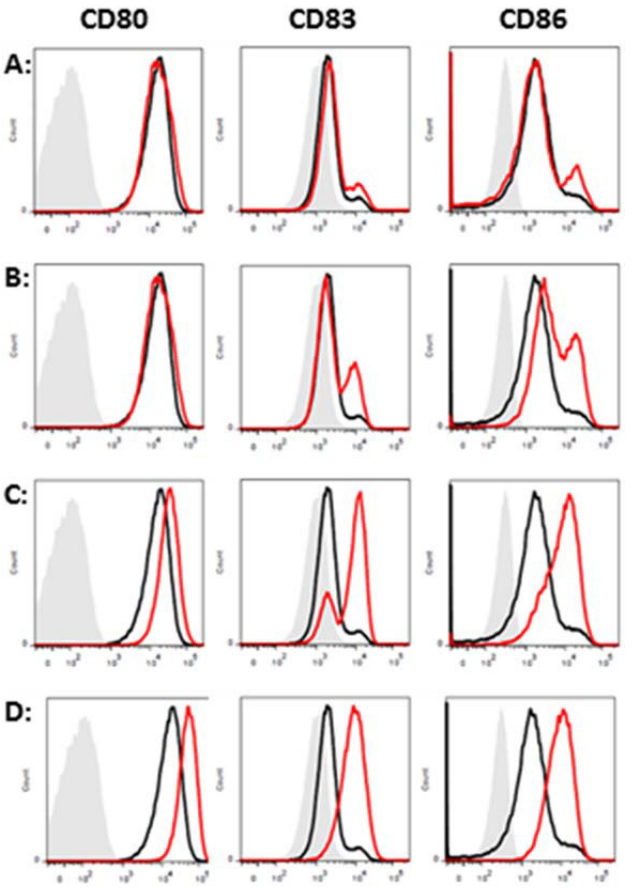

Figure. 2. Flow cytometry analysis of immune cell activation markers CD80, CD83 and CD86 from primary human dendritic cells exposed to A: rat tail collagen gels; B: Fibrewire® suture material; C: Material 2; D: Lipopolysaccharide positive control. ▬ = Untreated cells; ▬ = treated cells. All dendritic cells were taken from the same patient.

3. Discussion

During the evaluation of material 1, we demonstrated that the first manufacturing batch of material we received was highly cytocompatable (Figure 1A and B). However, the following manufacturing batch that we analysed was not conducive to cell growth (Figure 1C). Further analysis of this batch proved equally fruitless, as no cells were adhering to, or proliferating on the material surface.

Acknowledgements

This study was funded by an Auckland Medical Research Foundation Fellowship and the Maurice Wilkins Centre, NZ. These funding sources had no involvement in the study design; in the collection, analysis and interpretation data; in the writing of the report; nor in the decision to submit the paper for publication.

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