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Investigation of fibroblast and keratinocyte cell-scaffold interactions using a novel 3D cell culture system

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Abstract In this study we investigated the influence of fibre diameter and interfibre space in 3D scaffolds on cellular behaviour of human dermal fibroblasts and a human keratinocyte cell line (HaCaT cell). Electrospun aligned poly L-lactic acid fibres (2–10 μm) were bound to form fibres with a broad range of diameters (2–120 μm) and then constructed in a specifically designed 3D cell culture system. Human dermal fibroblasts were introduced to one end of the free-standing fibres using a fibrin clot and encouraged to 'walk the plank'. Under these conditions it was observed that a minimum fibre diameter of 10 μm for fibroblast adhesion and migration arose. A thin layer of electrospun viscose rayon scaffold fibres (diameter 30–50 μm, pore size 50–300 μm) was also constructed in the 3D cell culture system. After introduction to the scaffold using cells contained within a fibrin clot, fibroblasts were observed to stratify and also elongate between fibres in order to occupy voids. An interfibre span of up to 200 μm was possible by a single fibroblast, but more commonly void distances were spanned by cellular multilayering. In contrast, HaCaT keratinocytes cultured under identical conditions using viscose rayon scaffolds occupied very much smaller void distances of 50–80 μm predominantly by stratification.

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1 Introduction

Considerable research has been undertaken to develop tissue engineered scaffolds with well-defined architectures by modelling the extracellular matrix (ECM) in three dimensions (3D) [1, 2]. A number of synthetic and natural materials and many processing methods have been used to fabricate fibrous scaffolds with nano-, micro- and macro-scale fibre diameters [3–7]. However, it is still a complex matter of answering to what extent must a synthetic scaffold mimic the complexity of natural ECM in order to achieve a clinically useful engineered tissue, since there is a growing body of data showing that both scaffold architecture and scaffold chemistry can influence cellular response. Adherent cells are able to detect and interact with complex ECM architectures using filopodia, initially presenting a leading edge and then forming lamellipodia that adhere to ECM ligands via transmembrane integrin receptors [8–10]. If cellular adhesion is to a synthetic material, then a thin adsorbed layer of protein is usually responsible for mediating the cell-material interaction. *In vitro* cells have been found to respond to local macroscale, microscale, and nanoscale patterns of chemistry and topography [3], due to the differences in cell size and cell-matrix adhesion mechanisms [9, 11, 12]. It should be noted that cell behavior in 2D and 3D environments are also very different [13]. For example, fibroblasts have been found to form different focal adhesion structures and be more likely to proliferate in 3D than 2D culture [14] and epithelial cells also behave very differently in 3D environments compared to 2D culture [15].

Recent work from our group has shown that co-culture of three different types of skin cells (keratinocytes, fibroblasts and endothelial cells) in an electrospun 3D polystyrene (PS) scaffold devoid of biochemical cues can self-organise to a reasonable degree [16]. This suggests that 3D morphology

can sometimes dominate over material chemistry, suggesting that tissue engineered scaffolds may not have to exactly mimic the intended tissue in terms of molecular cues for such applications. In this study, we developed a simple and reproducible 3D cell culture system in which we could investigate the influence of 3D scaffold architecture on cellular behaviour. To obtain quantitative data on cell scaffold interactions, free standing poly L-lactic acid (PLLA) fibres of increasing diameters (2–120 μm) or a very thin layer of viscose rayon scaffold fibres (fibre sheet dimension: 5 mm $\times$10 mm $\times$ 0.1–0.2 mm) were mounted and suspended in cell culture media. Two different cell seeding methods were employed to introduce cells specifically at one end of the fibres or to the viscose rayon scaffold in a specific area. Once cells had adhered and proliferated to occupy the space immediately available to them in the cell seeding area the only option for continued growth was to migrate from the initial constrained space towards a scaffold fibre in proximal contact. Cells were then able to migrate and proliferate along fibres if a permissive diameter and/or material chemistry was present. The above device allowed us to investigate two very basic questions regarding the cell—fibre scaffold interaction: (1) what is the minimum fibre diameter for cell adhesion and migration when the cell only has the option of migrating along a fibre and (2) how wide a gap can be bridged by cells when fibres are suspended in culture medium?

2 Materials and methods

2.1 Cell culture

Normal human dermal fibroblasts were isolated and cultured under locally approved ethical guidelines (NHS, Sheffield, UK) in DMEM medium as described previously [16]. The HaCaT human keratinocyte cell line was kindly supplied by Professor N.E. Fusenig (Institute of Biochemistry, German Cancer Research Centre, Heildelberg, Germany), and cultured in a defined keratinocyte-serum free medium (Invitrogen, USA).

2.2 Electrospinning of aligned poly L-lactic acid fibres

Solutions of poly L-lactic acid (PLLA, M_w 250,000, Aldrich, UK) were prepared for electrospinning. Briefly, PLLA was dissolved in dichloromethane (DCM) under gentle stirring to obtain solutions of 7.5 and 10 wt%. Each polymer solution was delivered at a constant flow rate (3–5 ml/hr) via a syringe pump (WRI Aladdin-1000, UK) to a disposable 22GA blunt end needle (Camtel Ltd, UK). Upon applying a high voltage (17.5 kV) to the needle from a DC power supply (Alpha III Brandenburg, UK), a fluid jet was ejected toward a rapidly rotating (800 rpm) open cage collector at a distance of 20–25 cm. Over short collection times of a few seconds, aligned fibres with diameters of 2–10 μm were obtained. The fibres were lifted directly off the cage onto a glass slide in order to maintain alignment and then fabricated by binding and gluing several fibres together at both ends (1–2 mm) to achieve fibres with a very broad range of diameters (2–120 μm). The bound fibres were then mounted in a 3D cell culture system for cell culture. (The ends of the bound fibres were not used for cell culture to avoid the influence of the glue on material chemistry).

For fluorescence microscopy, the electrospun aligned PLLA fibres were fluorescently labelled with a rhodamine dye (Aldrich, UK). Briefly, a rhodamine stock solution was prepared in dichloromethane (DCM) to a final concentration of 1 wt% DCM. The rhodamine solution and the PLLA solution (1% (v/v)) were then mixed together with gentle stirring prior to electrospinning (detailed in Section 2.2).

2.3 Fabrication of a 3D cell culture system

As the schematic diagram (Fig. 1) illustrates, silicone sheets were used to mount aligned PLLA fibres with different diameters or a single layer of commercially available viscose rayon scaffold (Azowipe®: non-woven viscose rayon bonded with a styrene butadiene copolymer, Vernon-Carus Ltd, U.K., dimension: 5 mm $\times$ 10 mm $\times$ 0.1–0.2 mm). The 3D cell culture system with aligned fibres or viscose rayon scaffold

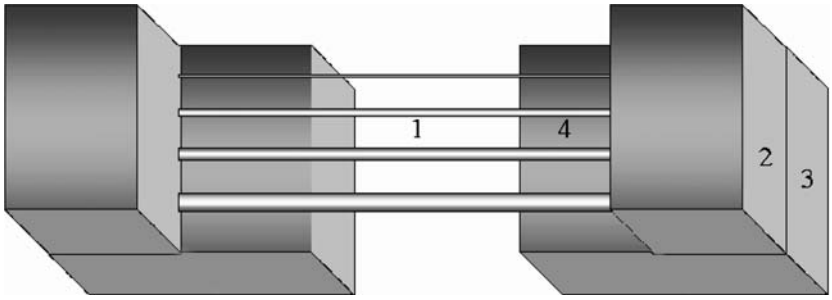

Fig. 1 Schematic diagram of the 3D cell culture system: (1) electrospun aligned PLLA fibres with different diameters (2–120 μm) or a single layer of viscose rayon scaffold (5 mm$\times$ 10 mm $\times$ 0.1–0.2 mm) were mounted between (2) an upper silicone block and (3) lower silicone block. After system sterilization, cells were seeded in (4) the cell seeding area near one end of the aligned fibre or viscose rayon scaffold using a fibrin clot or cell-material transplant

were cleaned with 70% ethanol, washed thoroughly with PBS (×3), sterilized water, dried and kept in tissue culture Petri dishes (9 cm in diameter). Petri dishes were used as cell culture chambers for experimentation. Cells were then seeded specifically near the end of aligned fibres in the cell seeding areas using a fibrin clot. For the viscose rayon scaffold, a small piece of fibrin clot containing cells was placed and fixed on one end of the scaffold in the cell seeding area (see 2.5 *cell-fibrin material for cell introduction* below). Cell culture was carried out by submerging the whole system in culture media. Once cells had grown out from the fibrin clot or the cell-material transplant and used up the space immediately available to them in the cell seeding area then the only option for continued cell migration and proliferation was to migrate along fibres or in scaffold with where an appropriate fibre diameters was present.

Since the migration length of single cells on aligned fibres or viscose rayon scaffold was hard to detect accurately in the culture medium during cell culture, phase contrast microscopy was used only for the general observation of cell proliferation in the fibrin clot, cell stratification in open pore structures and to monitor of system performance. To investigate the influence of fibre diameter and interfibre distances on cell behaviour more accurately, human dermal fibroblasts or HaCaT cells were seeded in the cell seeding area and cultured in the 3D cell culture system for two weeks. After cell culture, the cells that managed to attach and migrate on the aligned fibres or viscose rayon scaffold were fixed and stained with DAPI or phalloidin-TRITC to visualise nuclei and actin cytoskeletal fibres, respectively. Fluorescence microscopy was employed to detect single cells (using DAPI / phalloidin-TRITC fluorescence) and fibres (using rhodamine for PLLA and relying on the autofluorescence from viscose rayon fibres). Fibres with adhered cells were labelled as "cells attached and migrated", while the fibres without adhered cells were labelled as "cell failed to attach and migrate". To quantify the effects of inter-fibre distances, fluorescent micrographs of open pore structures with different sizes were taken and analysed using image analysis software (Openlab 4.0.2 and Volocity 3.0.2, Improvision, UK). The widths of open pore structures in each micrograph were measured and recorded. Gaps that were spanned by cells were labelled as "gap filled", while gaps that were not spanned by cells were labelled as "gap not filled".

2.4 Cell seeding using a fibrin clot

A fibrin clot was used to seed cells at a particular position in the 3D culture system, as follows: A cell suspension was prepared by mixing fibrinogen (3.5 mg/ml), thrombin (10 units/ml) and cells (5×10^5 cells/ml) together. 20 μm drops of the suspension were transferred prior to clotting to the cell seeding area located adjacent to the aligned fibres. The chamber was then kept in a tissue culture incubator (37°C and 5%CO_2/ 95% air) for 20–40 min to allow fibrinogen polymerisation. Culture medium was then added to the chamber to ensuring that the fibrin gel and PLLA/viscose rayon fibres were submerged cell culture.

2.5 Preparation of a cell-material scaffold for cell introduction

Stainless steel rings were employed to seed cells on top of 4–6 layers of stacked viscose rayon scaffold in 6 well tissue culture plates as described previously [16]. After seeding, scaffolds plus cells were incubated in 3 ml of medium in a tissue culture incubator (37°C and 5% CO_2) for 6 days and refed every 2–3 days. Then each layer of the scaffold containg seeded cells was separated and transferred into each well of a 6-well tissue culture plate containing culture medium. After confirming the presence of cells by bright field light microscopy, each scaffold layer was washed thoroughly with PBS to remove non-attached cells and then used as a cell-material device to deliver cells at one end of a separate viscose rayon scaffold in the 3D cell culture system. The device was brought in to physical contact at one end of the PLLA/viscose rayon fibres located immediately within the cell seeding area.

2.6 Fluorescence microscopy

Fluorescence microscopy was performed to determine the location of cells attached on aligned fibres by staining the nuclei with DAPI (300 nM, Vector Laboratories Inc., Ca, USA) and for F-actin with phalloidin-TRITC (25 ug/ml, P195, Sigma-Aldrich). In brief, after removal of the culture medium, the whole 3D cell culture system with aligned fibres or a viscose rayon scaffold in Petri dishes were washed gently with PBS (×3). The cells were fixed in 4% (w/v) paraformaldehyde for 30 minutes and labelled with DAPI and phalloidin-TRITC for 1 h. After a final wash (×3 PBS), epifluorescence images of cell-attached fibres or open pore structures with different sizes were taken directly without disassembling the cell culture system (using an ImageXpress^{TM}, AXON, USA) at $\lambda_{ex} = 358$ nm, $\lambda_{em} = 461$ nm (for DAPI/nuclei visualization), and $\lambda_{ex} = 550$ nm, $\lambda_{em} = 650$ nm (for rhodamine/scaffold or F-actin visualization). Cell orientation, cell outgrowth, fibre diameter and the widths of open pore structures were then analysed and measured from the fluorescent micrographs using image analysis software (Openlab 4.0.2 and Volocity 3.0.2, Improvision, UK).

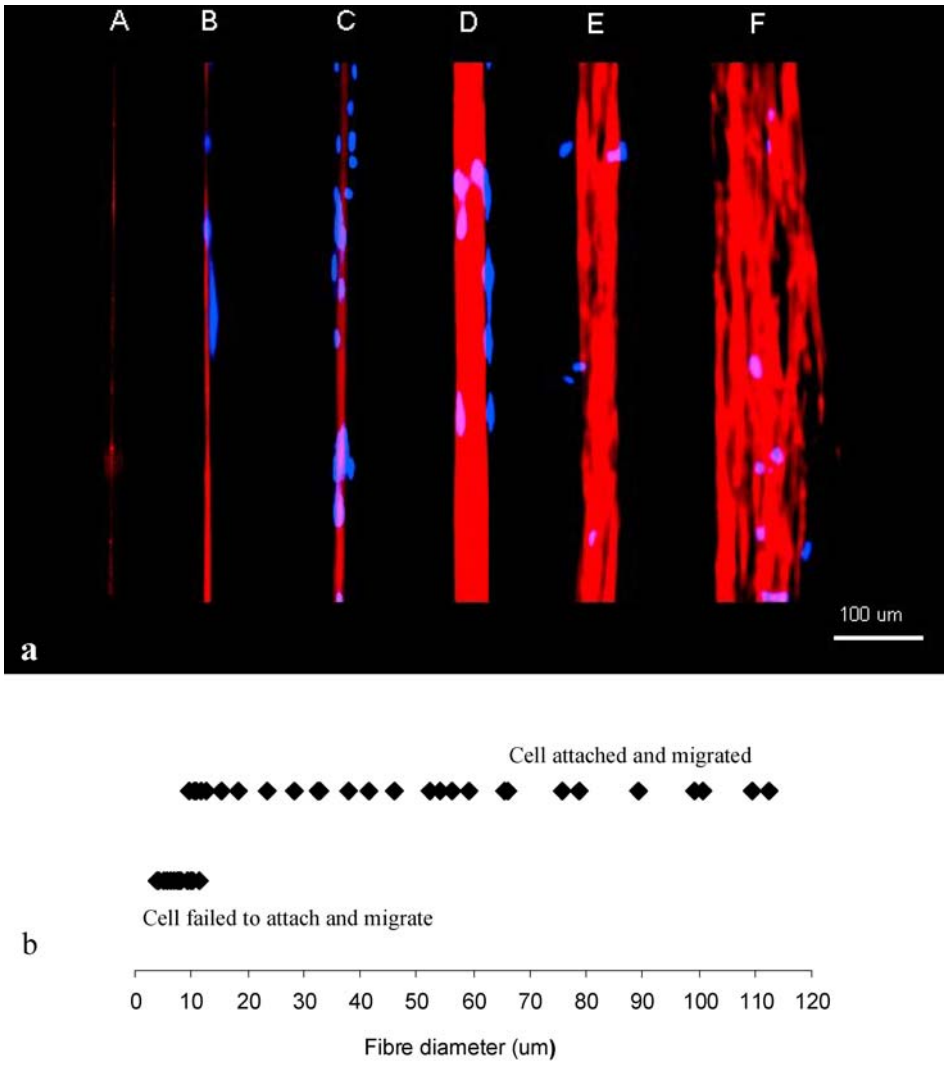

Fig. 2 (a) Fluorescent micrographs of human dermal fibroblasts cultured in the 3D cell culture system with electrospun aligned PLLA fibres with different diameters for 2 weeks. Each symbol represents a single fibre examined over two weeks. Scale bars are 100 μm. (b) A summary of the effect of fibre diameter on the ability of fibroblasts to attach and migrate on PLLA fibres cultured in the 3D cell culture system for 2 weeks. Fibres that supported cell adhesion and migration were labelled as *cell attached and migrated*, while fibres that did not were labelled as *cell failed to attach and migrate*

3 Results

3.1 Culture of human dermal fibroblasts on aligned PLLA fibres

Aligned PLLA fibres with different diameters (2–120 μm) were mounted in the 3D cell culture system and human dermal fibroblasts were then seeded in the cell seeding area (in a small fibrin clot) near the end of the aligned fibres. The whole system was then submerged in DMEM media supplemented with 10% FCS for cell culture. During cell culture, phase contrast light microscopy was used to identify that human dermal fibroblasts migrated out from the fibrin clot, and then migrated along immediately adjacent aligned PLLA fibres of appropriate diameters. After two weeks in culture, cells were fixed and stained with DAPI to enable detection by fluorescence microscopy. Systematic evaluation of fibres with different diameters demonstrated that human dermal fibroblasts had a minimum fibre diameter preference of 10 μm for both attachment and migration (illustrated in Fig. 2(a) and (b)).

3.2 Culture of human dermal fibroblasts on a viscose rayon scaffold

A cell-material scaffold initially constructed containing human dermal fibroblasts was placed at one end of a separate free-standing viscose rayon scaffold in the cell seeding area of the 3D culture system. During cell culture, human dermal fibroblasts were observed to migrate from the material 'transplant' onto the free standing viscose rayon scaffold and migrate along the fibres observed under brightfield light microscopy. After two weeks in culture, cells were fixed and stained with phalloidin-TRITC to visualise the degree of actin organisation. Due to the auto-fluorescence

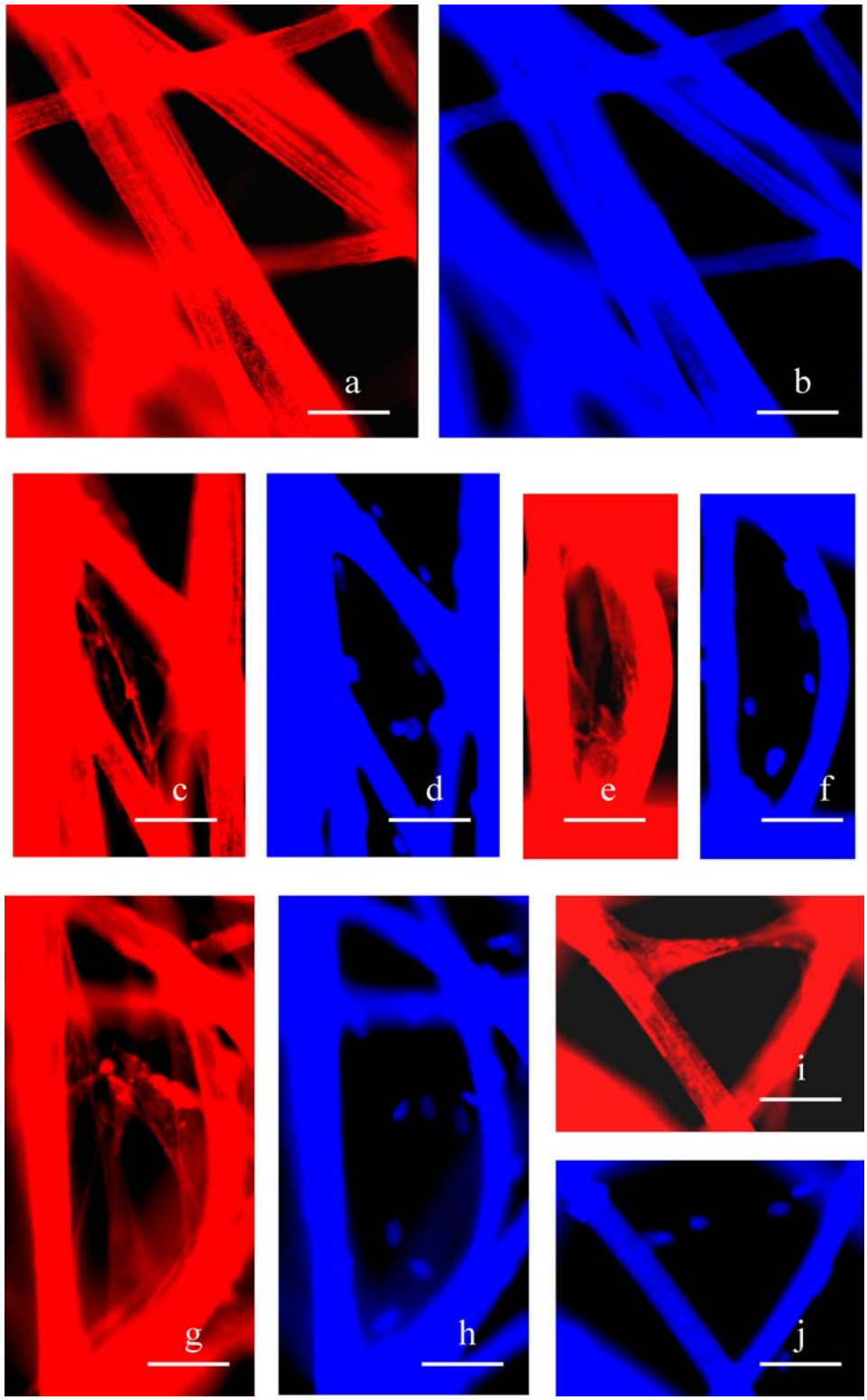

Fig. 3 Fluorescent micrographs of a viscose rayon negative control scaffold taken at (a) $\lambda_{ex} = 550$ nm, $\lambda_{em} = 650$ nm (red) and (b) $\lambda_{ex} = 358$ nm, $\lambda_{em} = 461$ nm (blue). Fluorescent micrographs of human dermal fibroblasts cultured in the 3D cell culture system with viscose rayon scaffold for 2 weeks in DMEM medium supplemented with 10% FCS. The cells were stained with (c, e, g, i) phalloidin-TRITC to identify F-actin filaments (red) and (d, f, h, j) DAPI to identify nuclei (blue). Scale bars are 100 μm

of the viscose rayon scaffold (shown in Fig. 3(a) and (b)) we did not need to use a staining procedure to identify these fibres. Fluorescence microscopy allowed us to identify that when cells encountered a fibre intersection they could form a multilayer structure and in so doing, occupy spaces between fibres (as illustrated in Fig. 3(c)–(h)). Single fibroblast cells and small fibroblast colonies were also observed to span fibres by taking on a stretched or elongated morphology (Fig. 3(i) and (j)). The maximum distance that fibroblasts could bridge between fibres over

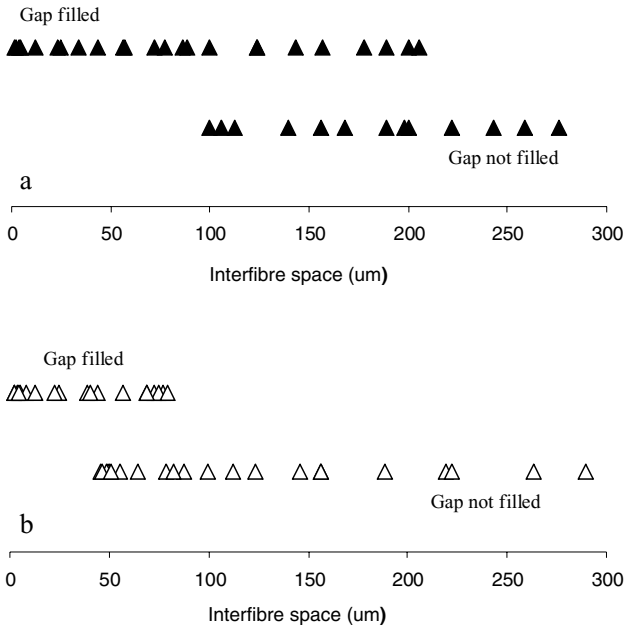

Fig. 4 Summary of the effect of interfibre distance on the ability of (a) human dermal fibroblasts and (b) HaCaT keratinocytes to bridge the gaps between fibres when cultured on viscose rayon scaffold in the 3D cell culture system for 2 weeks. The voids that spanned by cells were labelled with "Gap filled", while the voids that not spanned by cells were labelled with "Gap not filled"

a two week culture period was approximately 200 μm (Fig. 4(a)). The observation of fibroblast stratification or overlapping suggests a coordinated behaviour by groups of cells, as has been described previously (Engelmayr et al. [17]), rather than the behaviour of contact guidance on structured surfaces, which can affect individual cells [18].

3.3 Culture of HaCaT cells on viscose rayon scaffolds

A cell-material scaffold initially containing HaCaT keratinocytes was also prepared and physically located at one end of a viscose rayon scaffold and cultured in a defined keratinocyte serum free medium for two weeks. Fluorescence microscopy enabled us to observed that HaCaT cells could attach and migrate on single fibres or bundles of viscose rayon scaffold fibres as illustrated in Fig. 5(a)–(d). HaCaT keratinocytes were found to proliferate mainly at the intersecting points of fibres as shown in Fig. 5(e)–(f) and also able to bridge smaller gaps between fibres compared with fibroblasts (maximum gap distance 50–80 μm, summarized in Fig. 4(b)) through stratification (Fig. 5(g) and (h)). However, no single HaCaT keratinocyte cell was observed to span between fibres by adopting a stretched or elongated phenotype, as frequently observed with human dermal fibroblast cells.

4 Discussion

The extent to which a natural extracellular matrix (ECM) is needed in order to obtain an appropriate cellular behaviour necessary for the integration of cells into the body, and in turn its value for effective repair is a great challenge in developing tissue engineered scaffolds. A number of variables need to be considered when looking at the complex interactions between cells and 3D scaffolds. It is well established that cells can respond to surface chemistry and topography at the nano-, micro- and macro-scale [3]. Furthermore different cells have different properties in this respect due to several parameters including size, adhesion molecule expression or filopodia activity etc [11, 12]. The behaviour of a given cell type is also observed to change significantly when cultured in a 3D scaffold compared to culture on a conventional 2D surface [13–15]. Therefore, suitable 3D cell culture systems are important in order to investigate the interaction between a given cell type and its 3D environment. It should also be emphasized that the extent of cell-scaffold interaction is influenced by a number of material-independent parameters including cell density and culture conditions, especially at very high densities that extend well beyond monolayer culture [9]. In general terms, the delivery of cells in to a construct is not precisely controlled, but more often arises via an initial random introduction of cells on to a 2D surface or in to a 3D scaffold [19–21].

In the current research, a novel 3D system was designed to control and direct the introduction of human dermal fibroblasts and HaCaT keratinocytes in to a 3D culture environment for the subsequent investigation of cell-scaffold interactions. Cells were introduced to aligned PLLA fibres with controllable diameters (2–120 μm), or to a thin layer of a viscose rayon scaffold with fibre diameters of 30–50 μm and pore sizes of 50–300 μm). The scaffolds were then mounted and suspended in culture medium (as opposed to being placed on an underlying substrate). The influence of surfaces other than those being investigated was therefore excluded. The method employed to introduce cells to PLLA fibres or a viscose rayon scaffold involved creating a 'holding bay' by forming a cell seeding area. After cells had proliferated to occupy this region they were then able to migrate on to the adjacent suspended fibres or viscose rayon scaffold. During culture, brightfield light microscopy was used regularly to check cell growth and monitor the performance of the whole system. After two weeks in cell culture, the cell scaffold interaction was investigated qualitatively by fluorescence microscopy, and analyses performed

Our research suggests that the length scale of 3D scaffolds is an important criterion for the growth of cells when using electrospun fibres. The minimum PLLA fibre diameter for human dermal fibroblast cell attachment and migration was 10 μm. Below this diameter no fibroblast cells were

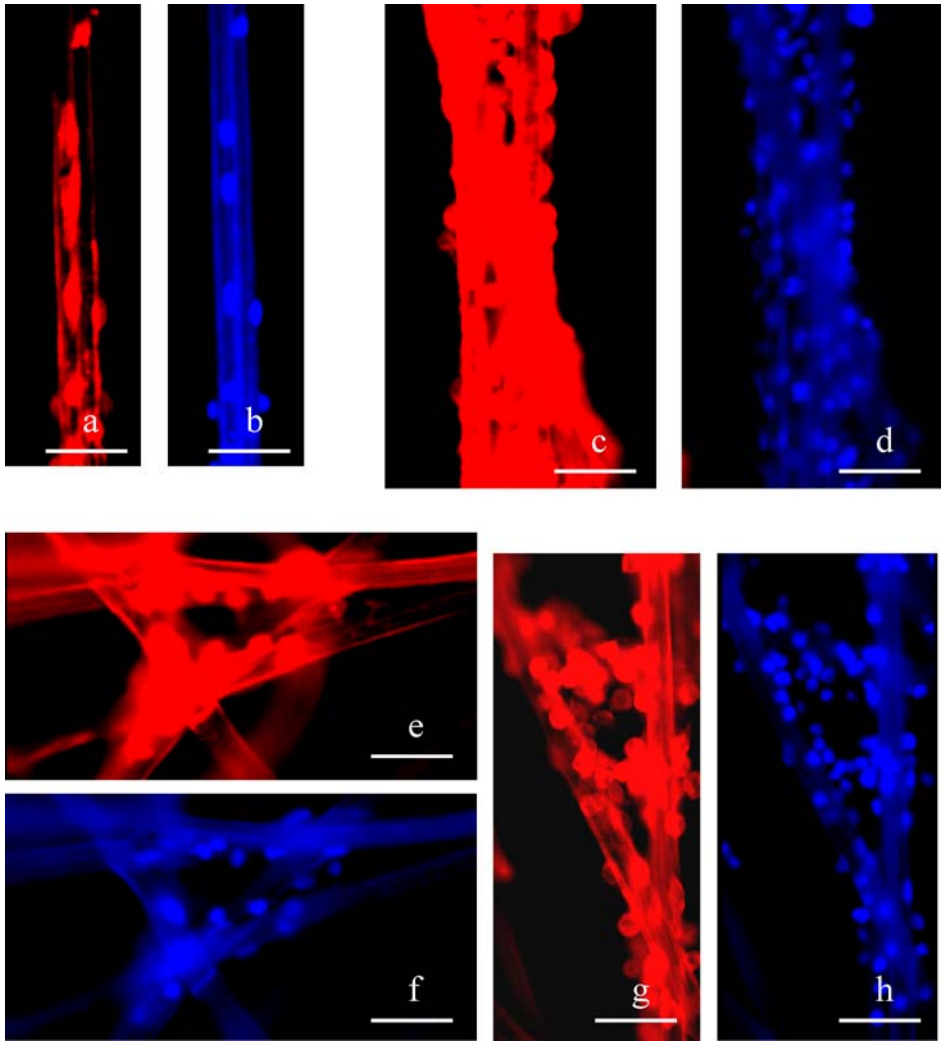

Fig. 5 Fluorescent micrographs of HaCaT cells cultured in the 3D cell culture system with viscose rayon scaffold using a defined keratinocyte serum free medium for 2 weeks. The HaCaT cells were stained with (a, c, e, g) phalloidin-TRITC (red) and (b, d, f, h) DAPI (blue). Scale bars are 100 μm

observed to adhere to fibres, although cells were observed in the culture chamber they were not specifically attached to fibres. The behaviour of cells on viscose rayon scaffolds indicated that the maximum gap that fibroblasts could bridge between fibres was approximately 200 μm. Interestingly, human dermal fibroblasts and HaCaT keratinocytes were observed to occupy the open pore structures differently. HaCaT cells bridged interfibre spaces mainly by a process of stratification. It was also noted that in contrast with fibroblasts, keratinocytes were rounder in shape and smaller in size (10–20 μm in diameter). Consequently, the maximum inter-fibre gap size for HaCaT cells to span was between 50 and 80 μm. Indeed, single fibroblasts were observed to span considerable distances (150–200 μm) and colonies were observed to stretch even further and elongate between fibres in order to span the voids.

The approach we have adopted to look at fibre diameter and distance between fibres can be used to systematically investigate or compare permissive cellular properties and limitations of novel cell-scaffold interactions. It can also be extended to study fibres of different chemistries and to incorporate nano-features or nano-patterning into micron diameter fibres to determine to what extent nano-scale features in micro-diameter fibre scaffolds can influence cell biology.

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