Morphometry and Tissue Engineering Studies of Keloidal Cells

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Abstract: This study includes new methods to study keloid and hypertrophic scars, which are morphometric studies and the use of tissue engineering technique; this may lead to find a new alternative way in Keloid management. Morphometric analysis reflects the nature of the keloidal cells, which appear with different dimension compared with normal fibroblasts and hypertrophic cells. The tissue engineering results indicate that there is significant increase in the cell length when cultured on topography. Morphometric analysis indicate that keloidal cells shorter than normal fibroblasts, while keloidal cells adhere to grooved topography appeared longer than normal fibroblasts. This revealed that keloidal cells response to topography by aligned and increase in length.

Keywords: Morphometry, tissue engineering, keloid, fibroblasts

INTRODUCTION

Wound healing abnormalities are among the greatest cause of human deformity and disability, which can produce functional and psychological problems (Bertolami and Bronson, 1990; Kosit et al., 2000).

The nature of the interactions of a cell with other cells and with a substrate is an important problem in biology. One method of studying these interactions is to grow cells in tissue culture. When mammalian cells are grown in vitro, most cells do not divide unless they are attached to a solid surface. This surface is commonly covered with an adsorbed layer of serum protein (Pettinini et al., 1958; Saxen and Pettinini, 1965; Gaeber and Ward, 1978). Therefore, it is possible to grow mammalian cells in vitro in the laboratory, where the cells act more like independent organisms with a generation time of approximately 24 h. For many purposes, it is sufficient, cheaper and more practical to study cells in tissue culture rather than in an intact animal (Carrel, 1912; Khorshid, 2001; Khorshid and Mushref, 2005).

The use of tissue or cell culture for the study of keloid scars involves isolating fibroblasts from excised keloid tissue and propagating them in the laboratory using exogenous nutrients and growth media (Cohen, 1990). This technique allows for manipulation of specific experimental variables. Individual biological pathways, processes and molecules can be examined. Cells are isolated from the physiological environment, thus eliminating many confounding variables. Isolating cells from their environment can also have limitation. Required to propagate the cells are ideal and are often unlike physiological conditions, i.e., abundance of nutrients, ideal pH, temperature and gases. Most keloid seare tissue culture models involve the use of serum. Yet, the wound environment that gives rise to keloid scars is one exposed to the biological factors contained (Brunette, 1986a).

On other hand, biological cells are also strongly influenced by the topography of the surface on which they live, both in cell culture and in vivo. They are guided along micron sized grooves and change their shape becoming more elongated (Brunette et al., 1983; Clark et al., 1990, 1991; Wojciak-Stothard et al., 1996). These effects can be used for cellular engineering to determine the behavior of cells and in particular to make prostheses for medical purposes (Wilkinson et al., 1998).
These are important concepts for exploitation in cell engineering. According to Wilkinson et al. (1998), cell engineering can be defined as the use of cells and parts of cell to build systems useful for biomedical purposes and for fundamental research.

In recent years, the microfabrication technologies have been employed by biologists to examine cell behavior (Burnette, 1986a, b; Dunn and Brown, 1986; Clark et al., 1987, 1990, 1991; Hirono et al., 1988; Wood, 1988). Therefore, topographic cues have been shown by various studies to guide cell growth, migration, guidance and orientation (Clark et al., 1987; Wojciak-Stothard et al., 1995). Although the chemistry of the material could be affecting the behavior of the cells (Curtis and Clark, 1990), it is necessary to examine the types and the sizes of structures that affect cell behavior in vitro to determine a mechanism for guidance and to show whether or not topographical environments found in nature could act as cues (Clark et al., 1990). The behavior of cells on multiple parallel grooved substrata is now well developed and when compared with the behavior of the same cell types on flat surfaces shows a marked difference in responses (Curtis and Wilkinson, 1997). Wojciak et al. (1995) enhanced the use of multiple grooved substrata to facilitate cell movement and to promote tendons healing in vitro.

The aim of the present study was to investigate application of new methods to study the in vitro behaviors of keloidal and hypertrophic cells using morphometric study and tissue engineering technique. If these methods worked out this may lead to find a new alternative way in keloid management.

The idea is to facilitate the closest collaboration between biologists and plastic surgeons in developing new treatments for keloids by studying the keloidal cells behaviour.

**MATERIALS AND METHODS**

**Minimal Essential Medium MEM (10% Fetal Calf Serum)**

MEM is a multipurpose medium that was used for cultivation of mammalian cells (Pollared and Walker, 1989).

Phosphate Buffered Saline (PBS) is a Phosphate-Buffered physiological Saline solution. Calcium and Magnesium free Solution (Pollared and Walker, 1989; Khorshid, 2001).


EDTA is Ethylenediaminetetra acetic acid disodium salt (Pollared and Walker, 1989; Khorshid, 2001).

**Human Samples**

The informed consent was obtained from all subjects. The study was approved by King Abdul Aziz University ethical committee.

**Human Keloid**

Human keloid samples were obtained from patients, were used as raw materials for isolating human fibroblasts from keloid and normal skin. The keloid skin was obtained from scars excised during plastic surgery performed at King Abdul Aziz University Hospital.

**Human Skin Fibroblast Cells**

The normal human specimens (human skin fibroblasts) were obtained from King Abdul Aziz University Hospital, after circumcision operations. The specimens transported immediately after excision in previously prepared bottles of MEM media (transport time between operating theater and our laboratory approximately 5 min).

**Tissue Culture Experiment**

The samples were cut into small fragments, minced and gently agitated in trypsin solution at a concentration of (0.25, 0.1% glucose and 0.02% EDTA) for 15 min. Trypsin action was quenched by
MEM when intercellular separation was seen. The supernatant suspension containing the dissociated cells was removed and centrifuged at 100 x g for 10 min at 4°C, cells were re-suspended in MEM containing 20% Fetal Calf Serum (FCS) heat inactivated (56°C for 30 min). Cells were adjusted to 1x10^6 cells mL^-1 and plated into tissue culture flask 25 cm² then incubated in a humidified incubator in an atmosphere of 5% CO₂ at 37°C.

Cells were grown in 25 cm² polystyrene flasks and passage biweekly. Cells suspended in PBS, centrifuged, re-suspended in culture medium (Pollared and Walker, 1989). Each group of cells was cultured to a suitable number of cells using MEM media.

One gram Coomassie Brilliant blue R- 250 dissolved in 1L of 500 mL methanol /500 mL water and 70 mL acetic acid was used to stain cells for the light microscopy examination and imaging.

Morphometric analysis recorded for cultured cells after fixation and staining during the tissue culture experiments by measuring the length and greater width of the examined cells using an ocular micrometer, measurements were compared with control and analyzed.

**Tissue Engineering Study**

The work in this project deals with the effect of topography on cell behavior on plane and multiple parallel grooved substrata. All grooved structures were fabricated from polycaprolactone (PCL).

Microgrooved structures with grating width a bout 5-10 μm made by Electronics and Electrical Engineering Department in Glasgow University.

All these structures were cleaned by soaking in absolute methanol for one week then sterilized in 70% ethanol 10 min followed by a rinse in reverse osmosis (RO) water that replaced by media before using.

Each group of cells were plated onto multiple grooved substrata with groove width 5-10 μm and incubated in MEM at 37°C for 24 h. Cells were fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1xPBS each for 5 min. Then cells were stained with coomassie blue for 5-10 min followed by repeated washing with tap water.

Images typically were recorded using a digital camera and saved in computer for studying later.

**RESULTS**

**Morphometry of the Cells**

Measurement of the length of about 80 cells from different types of cells (keloid, hypertrophic and normal) after 24 h for three tissue culture experiments shows that the keloid cells which adhere to normal (planner) petri dishes appear shorter than normal cells but the hypertrophic are the shortest. The differences between normal and keloidal cells or the normal and the hypertrophic cells length are significant. While the difference between hypertrophic and keloidal cells length is not significant, Table 1

Measurements of the widths of the studied cells indicated that keloidal cells had wide cell body than normal fibroblasts, but hypertrophic fibroblasts appear with less width than keloid or normal fibroblasts. Whereas there are no significant differences between keloidal and normal cell length, Table 2. This indicates that the keloidal cells spreading more than normal fibroblasts with more process.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
<th>St. dev.</th>
<th>p-value</th>
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<tr>
<td>Keloid</td>
<td>39.6</td>
<td>35.5</td>
<td>27.4</td>
<td>29.0</td>
<td>5.680391</td>
<td>0.012</td>
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<tr>
<td>Hypertrophic</td>
<td>28.6</td>
<td>25.3</td>
<td>32.2</td>
<td>24.9</td>
<td>3.390293</td>
<td>0.002</td>
</tr>
<tr>
<td>Normal</td>
<td>47.2</td>
<td>44.1</td>
<td>44.0</td>
<td>39.9</td>
<td>2.594439</td>
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Table 1: Diversity in cell lengths in different scar types
Tissue Engineering Study

A Study of The Morphology of Keloidal Cells Grown On 5-10 μm Groove Ridges

The aim of this experiment is to examine the morphological and physical properties (length, alignment, motility etc.) of the cells in reaction to grooved substratum. The reaction of keloidal cells to topography is not reduced as other abnormal cells, so the average of length of cells increased as the incubation time is increased for all experiments. Cells grown on planner surfaces gave reverse results. Also keloid fibroblasts cells aligned to the topography shape comparing with cells grown on planner surfaces as shown in the related Fig. 1-6. Experiments were done on 5-10 μm groove ridges and on planar controls.

Keloidal fibroblasts that were grown on fabricated structures with grating width of 10 μm appear longer than normal fibroblasts (Fig. 1 and 2). The keloidal cells aligned with grooved structure, which indicated the response of the cytoskeleton of cells to the shape of the topography but keloidal cells appear with multiple processes that revealed the high motility of the cells (Fig. 3 and 4).

These results indicate that there is significant increase in the cell length when cultured on topography.

It is clearly observed from these results that there is a difference between control and experimental cells in the morphology of the cells rather than the number of cells. The control cells appear spread with well-developed lamellipodia. While the keloidal cells appear have more ability to spread and grow.

The number of the keloidal fibroblasts was lesser than normal fibroblasts on all examined structures (Fig. 5 and 6).

Comparison of the cultures suggests ways that topography could determine cell shape. Cell length increased in keloidal fibroblasts see for example picture 1 and 2. This study indicates that the reaction

<table>
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<th>Table 2: Diversity in cell width in different cell types</th>
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<td>Mean of width 20 cells</td>
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<tr>
<td>Exp</td>
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<td>-----</td>
</tr>
<tr>
<td>Keloid</td>
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<tr>
<td>Hypertrophic</td>
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<td>Normal</td>
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Fig. 1: Light microscopy image (10x-Objective) of Keloidal fibroblasts grown on PCL grooved topography with depth 5-8 μm and 5-10 μm width in MEFM media for 24 h. Fixed and stained with Coomassie blue. Scale bars = 60 μm
Fig. 2: Light microscopy image (10x Objective) of Normal fibroblasts grown on PCL grooved topography with depth 5-8 and 5-10 μm width in MEM media for 24 h. Fixed and stained with Coomassie blue. Scale bars = 60 μm.

Fig. 3: Light microscopy image (20x Objective) of Keloidal fibroblasts grown on PCL grooved topography with depth 5-8 and 5-10 μm width in MEM media for 24 h. Fixed and stained with Coomassie blue. Scale bars = 200 μm.

Fig. 4: Light microscopy image (20x Objective) of Normal fibroblasts grown on PCL grooved topography with depth 5-8 and 5-10 μm width in MEM media for 24 h. Fixed and stained with Coomassie blue. Scale bars = 200 μm.
Fig. 5: Light microscopy image (40x-Objective) of Keloidal fibroblasts grown on PCL grooved topography with depth 5-8 and 5-10 µm width in MEM media for 24 h. Fixed and stained with Coomassie blue. Scale bars = 500 µm.

Fig. 6: Light microscopy image (40x-Objective) of Normal fibroblasts grown on PCL grooved topography with depth 5-8 and 5-10 µm width in MEM media for 24 h. Fixed and stained with Coomassie blue. Scale bars = 500 µm.

Fig. 7: Light microscopy image (a. 40x and b. 20x Objective) of normal fibroblasts grown on planer surface in MEM media for 24 h. Fixed and stained with Coomassie blue.
to topography is increased in keloidal tissues of all structures used in the experiment when compared with the length of keloidal cells that were grown on planar surfaces (Fig. 7) and that can be tested by further experiments.

**DISCUSSION**

Tissue culture studies are less expensive compared with studies performed using animal models. One limitation of using keloid tissue culture is the inability to test prophylactic treatments. Establishing a keloid cell culture requires preexisting keloid fibroblasts. Tissue culture is not useful for predicting the efficacy of a treatment in human. A model that includes only the fibroblasts (an isolated component of the entire wound-healing system) cannot entirely reproduce the complexities of human tissue repair.

Studies designed to verify or elucidate pathophysiological steps in keloid formation or a treatment's mechanism of action can be accomplished effectively with the cell culture system because of the ease of manipulation that they permit. They can also be used for a particular agent to aid in establishing a dose range that would achieve therapeutic efficacy in human (Hillmer and Maceoed, 2002). In this project we used Tissue Engineering method to study the keloidal fibroblasts, which can be used in mechanical management of keloid.

Tissue Engineering part of the project had studied the effects of the fibrous proliferations of dermal cells on the reaction of cells to topography. The reasons for doing this are to:

- Consider the role of fibrosis in cellular reaction to topography.
- Look at cells in an environment resembling intercellular conditions where cells adhere to tissue fibers (eg collagen), extracellular matrix and neighboring cells.
- Study the condition that affects the correct growth of cells in the laboratory first and then inside the body.
- Control cell adhesion, position and orientation which are vital to give correct growth and cell function.
- Study conditions (topographical cues) that allow making constructs from living cells outside the body for subsequent implantation.

The topography around cells may be that of surrounding cells, intercellular materials or biomaterials. The reactions to topography include cell orientation, rates of movement and activation of the cells (Curtis and Wilkinson, 1997). A few studies have reported that the changes in cell shape can influence the gene expression (Chou et al., 1995), therefore it would seem reasonable to investigate the effect of topography on the behavior of keloid fibroblasts.

The effects of trauma on reaction of cells to topography appear to be linked to changes in cell morphology, spreading, movement and on theoretical grounds to mechanical interactions with the substrate.

The major result of the tissue engineering study here is that the morphological reactions to topography and the movement of cells on topography were both markedly increased when the cells is keloidal. These results indicated that the behavior of the same cell types (fibroblasts) on grooved surfaces shows marked differences in responses that related to the affecting of cells by trauma. The reaction of cells to topography is not reduced because the average of length of cells increased in the keloidal cells, which revealed that the topography affect the keloidal cell morphology. The keloidal cells were longer and very well spread, low in number and large in size.

Cells are able to orient themselves in response to external signals. The sensitivity of keloidal cells to signal gradients may be extremely high. Cell polarisation initiated by gradients of an external signal is then stabilised by global reorganisation of the cytoskeleton. Change in the external conditions of cells
may be affecting the cell polarisation by affecting the redistribution of pseudopodia due to the reorganisation and orientation the whole cytoskeleton. This may explain the disorientation of keloidal cells inside the body tissues, where the cells align to the extracellular fibers as collagen. The number of the keloidal fibroblasts was lesser than normal fibroblasts on all examined structures. This also confirmed by our previous work used cell count method (Khorshid, 2006, 2006).

Previously many worker suggested that many types of cells react to microtopography and nanotopography by changes in important cell behaviour processes including: cell morphology, adhesion, changes in movement, contact guidance and tissue organization (Chehroudi and Brunette, 1995; Brunette et al., 1988; Curtis and Clark, 1990; Singhvi et al., 1992), activation of tyrosine kinases (Nobes et al., 1995; Wojciak-Stothard et al., 1996), condensation of actin cytoskeleton (Rovensky and Samoilov, 1994; Oakley and Brunette, 1993) and changes in gene expression (Ohara and Buck, 1979; Oakley and Brunette, 1995; Webb et al., 1995; Maylo et al., 1994).

Since it is easy to observe the morphology of the cell, most of the data here refers simply to morphology and its concomitant orientation.

Other workers suggest that cells do not conform to many topographies as closely as they would to planar surfaces (Dunn and Brown, 1986; Brunette, 1986; Clark et al., 1987; Curtis and Clark, 1990; Oakley and Brunette, 1993). The reason for this could be that the cells are under tension between attachments to groove edges and that this tension pulls the plasmalemma away from the groove bottom. In present experiment we found that the normal fibroblasts comfort more than keloidal fibroblasts to planar surfaces, whereas keloidal cells appear to react more with grating surfaces. This difference between reactions of keloidal cells with different surfaces could explain by the activity of cytoskeleton and the response of this cytoskeleton to the shape of the external feature (grating), which assimilate the extracellular matrix ECM and the abundances of collagen fibers in keloidal tissue in the human body.

The growth and development of an entire population society of cells could be controlled through structural alterations of ECM that produce associated changes in cell shape and associated physical force redistributions (Ingber et al., 1981).

Ingber and Folkman (1987), stated that the extracellular matrix serves as a local solid state regulator of soluble growth factor action through its ability to modulate cell and nuclear structures. Another study from Machesky and Hall (1997), mentioned that adherence of cells to extracellular matrix mediated through integrins is essential for normal cell development and movement.

Madjid and Williams (1983), found that purified ECM molecules can modulate the growth and organisation of the capillary endothelial cells in vitro.

The individual collagen fibers are thickened, hyalinized and highly eosinophilic. The fibers are usually arranged in nodules or whorls (Marrey, 1993).

These suggestions seem important in the present study because the keloidal appearance could affect the thickness of the extracellular matrix inside the body. The matrix becomes increased in thickness by high viscous collagen or also molecules that interpenetration by adding or removing water and in turn affects the diffusion of molecules into/ or out of the cells and also could affect the adhesion of cells to the matrix, which is reflected on the cell movements.

Activation of cells by reaction to topography is highly significant and can be brought about by reactions such as the changes in cytoskeletal condensation produced by contact of a cell to topography (Oakley and Brunette, 1993; Wojciak et al., 1996).

Oakley and Brunette (1993), examined the sequence in which microtubules, focal contacts and microfilament bundles become aligned to the substratum topography. They seeded human gingival fibroblasts onto grooved titanium surfaces as well as onto control smooth surfaces. They used fluorescent phallacidin to stain actin filaments. They found that focal contacts were closely associated with the termination of actin microfilaments and microfilaments bundles became aligned, as well as the cell itself, with the grooves.
Actin filaments have been cast in major roles in the development of cell orientation and polarization (Dunn and Heath, 1979; Dunn and Brown, 1986; Oakley and Brunette, 1993). However, it could be argued that the actin alignment might be a determinant of cell orientation in our study, which needs more investigation.

CONCLUSION

This study indicates that the keloidal cells spreading more than normal fibroblasts with more process. The reaction of keloidal cells to topography is not reduced because the average length of cells increased, which revealed that the topography affect the keloidal cell morphology. The keloidal cells were longer and very well spread, low in number and large in size.

ACKNOWLEDGMENT

The investigator would like to thank the Scientific Research Council from King Abdul-Azziz University for the valuable financial support of this work by grant No. 004/424.

I would like to thank Dr. Sabah Mosheri, from Department of Plastic Surgery, Professor Yasir S. Jamal, professor of Pediatric and Plastic Surgery, KAUH, for providing human specimens and participating during scientific discussions; Dr. Inas Hamed from Physiology Department KAAU for revising this article; Dr. Niveen AbduhAzim from Medical Biology Department KAAU for revising this article and for her helpful suggestions during the course of this study; Mrs. Nagwa T. Heffny for excellent technical assistance.

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