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Grooved substrata facilitate *in vitro* healing of completely divided flexor tendons

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Multiple grooved substrata with groove depth 5 μm were found to facilitate the healing of completely divided rat flexor tendons *in vitro*. Sections of tendons cultured on plain substrata showed only partial healing with incompletely sealed epitenon layers and immature thin collagen fibres. Tendons cultured on patterned substrata healed with complete restoration of the epitenon layer and reconstitution of the internal structure of collagen fibres. Epitenon fibroblasts isolated from the surface of rat flexor tendons were shown to be more sensitive to topographical features than fibroblasts of the same size BHK fibroblasts. They remained more elongated and better aligned to the groove direction than BHK cells. Multiple grooved substrata facilitated epitenon cell movement. Cells were found to move with higher speed on patterned substrata than on plain substrata. In summary, we conclude that the use of multiple grooved substrata promotes tendon healing *in vitro* and may find application in clinical practice in tendon repair.

1. Introduction

Tendons are highly specialized structures which transmit the forces of muscle contraction to distant bones thereby producing the movement at joints. In order to fulfill this function, they must be strong, flexible and have the ability to glide. Their strength and flexibility is provided by a core of longitudinally arranged collagen bundles among which are scattered small numbers of tenocytes. Their ability to glide is provided by a coating layer of synovial cells known as epitenon cells. In sites where large gliding movements take place, for example in the fingers, the tendon is enclosed in a tightly fitting synovial sheath and lubrication is provided by a thin layer of synovial fluid.

When a tendon is injured, healing takes place by a proliferation of epitenon cells which migrate into the gap between the divided tendon ends and synthesize collagen which restores the strength and continuity of the whole structure [1]. Surgical repair is undertaken to facilitate this process by holding the tendon ends together with a relatively strong core suture and a fine circumferential suture to approximate epitenon. Immobilization in a relaxed position for 3–4 weeks or gentle passive mobilization permits healing with fibrous tissue in most injuries. As crosslinking begins to take place between the newly formed collagen bundles 3–4 weeks following the injury, full mobilization is commenced. However, two major complications can occur and produce poor results. First, a number of tendon repairs disrupt, usually at an early stage, resulting in a complete loss of function and requiring salvage surgery. Secondly, and much more commonly, fibrous adhesions may form between tendon and synovial sheath due to uncontrolled migration of epitenon and synovial cells into the synovial space, and prevent gliding movements [1, 2]. This causes considerable disability by producing loss of function and often stiffness and deformity in the affected joints.

Thus it would be beneficial to design methods of enhancing the orientation and guidance of epitenon cells between the ends of divided tendon in order to hasten and strengthen healing. In addition, systems which guide and orientate cells may reduce the haphazard migration of epitenon and synovial cells, thereby minimizing adhesion formation.

In cell biology, systems which can produce cell guidance have been well described by several authors [3–10]. The best known structures which orientate fibroblasts and induce their directional movement are multiple grooved substrata [3–12].

In this paper we used fused silica substrata with multiple grooves in tendon organ culture. The dynamics of tendon healing was compared on plain and patterned substrata. We also studied the sensitivity of epitenon cells to topographical features: cell elongation, orientation and speed of movement. Results are discussed in terms of application of patterned substrata in surgical practice.

2. Materials and methods

2.1. Substratum patterning

Fused silica samples (Multi-lab) were cut into 25 mm^2, 1 mm thick samples. The silica was cleaned by soaking in a solution of 3:1 sulphuric acid: hydrogen peroxide for 5–10 min at 60 °C followed by rinse in R.O. water, then blow dried with filtered air. The silica was coated

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with AZ 1400-31 photoresist (Shipley) by spinning on at 4000 rpm for 30 s followed by a soft bake at 90 °C for 30 min. This gave a resist thickness of 1.8 μm. The resist was then patterned by exposing to u.v. light, through a chrome mask patterned with the required grating pattern, using a mask aligner (HTG) for 10 s. The exposed resist was developed off by immersing the sample in a solution of 1:1 Shipley developer: R.O. water for 65–75 s followed by a rinse in R.O. water, then blown dry.

The samples were dry etched in a RIE Unit (Plasma Technology). After etching the residual resist was removed, and all samples blanket etched for 1 min.

2.2. Organ culture

Flexor tendons were isolated from the middle digit of the hind paw of male Sprague Dawley rats. Twelve 8-weeks-old rats were anaesthesized using halothane. Synovial sheath was removed and the tendons were divided and placed on to plain and patterned fused silica substrata (grooves 5 μm deep, 10 μm wide) so that the gap between two tendon ends was 0.5 mm wide. Tendons were placed in parallel to the direction of the grooves and pressed with a clean coverslip. Tendons were incubated in BHK culture medium (20 mM HEPES buffered Glasgow modified MEM (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 0.5% bicarbonate, 10% foetal calf serum (Gibco), 10% tryptose broth (Gibco), 2.85 mM glutamine, antibiotics) for 3 weeks. The medium was changed every 48 h. After 3 and 5 weeks tendons were used for frozen sections and histological staining. Some of the healing tendons were studied under a light scanning confocal microscope.

2.3. Cell culture

Epitenon fibroblasts. Rat epitenon fibroblasts were isolated from rat flexor tendons of male Sprague Dawley rats as described by Wòjciak and Crossan [18]. Briefly, in step 1, the synovial sheath was removed by incubation of tendons in 0.5% collagenase (Clostridiopeptidase A; EC 3.4.24.; Sigma Chemical Co., Poole, UK) for 10 min at 37 °C.

In step 2 tendons were incubated in trypsin/EDTA solution (trypsin, 300 BAEE (N α-benzoyl-L-arginine ethyl ester) U/ml; EDTA, 0.001 M EDTA) for 1.5, at 37 °C then the released cells were suspended in BHK21 medium and centrifuged at 200 g for 6 min. Cells were then resuspended in the culture medium (BHK21) and plated into 25 cm^2 Falcon culture flasks at cell density 2×10^5 cells/ml. For experiments they were used between 15 and 25 passages.

For experiments epitenon cells were plated on to plain and patterned fused silica substrates at cell density 2×10^4 cells/ml. After 24 h cells were washed in serum-free Hank's balanced salt solution and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 5 min. Then the cells were washed again in PBS, stained in Kenacid blue (Sigma, UK) (0.1% in water/methanol/acetic acid, 50:50:7) for 10 min, and washed three times in PBS. Then cell guidance was analysed using the image analysis system.

BHK fibroblasts. BHK (Baby hamster kidney cells) were routinely maintained in the laboratory in culture medium: Glasgow modified Eagle's Minimal Essential Medium (GMEM) (Gibco, UK) supplemented with 3 mM glutamate (Sigma Chemical Co., UK), 100 units ml^{-1} penicillin, 100 ug ml^{-1} streptomycin (Flow labs, UK), 10% tryptose phosphate broth (Gibco, UK) and 10% calf serum (Gibco, UK). Cells were plated on to the patterned substrata, cultured and analysed under the same conditions as epitenon cells.

2.4. Preparation of frozen sections of the tendon and histological staining

Frozen tissue sections were prepared as described by Aaron *et al.* [17]. Sections of healed tendons were then stained with haematoxylin and eosin.

2.5. Confocal scanning microscopy

Completely divided tendons cultured *in vitro* on plain and grooved substrata were examined under the Odyssey Model Laser Scanning Microscope-VSMD (Noran Instruments Inc) equipped with an Argon-ion laser, working with the MetaMorph Imaging System (Universal Imaging Corporation, 502, Brandywine Parkway, West Chester, PA 19380, USA). Non-stained tendons were examined under the reflected light, pinhole size 2 mm.

2.6. Image analysis system

Images from × 10 objective of a Leitz Ortholux microscope were digitized to 512 × 256 pixels and 64 grey shades with a Hamamatsu Vidicon C1000 camera and Archimedes digitizer (Watford). The image analysis program written by C. Edwards (Chemistry Department, Glasgow University) in Acorn Risc machine assembler language for an Acorn Archimedes 310 microcomputer, was used to calculate cell spreading area, elongation and orientation. These parameters were calculated as described in [9].

2.7. Measurement of the speed of cell movement

Video time lapse cinematography was used to analyse the speed of movement of epitenon cells on plain and patterned substratum. Cells plated on to patterned fused silica substratum (grooves 5 μm deep, 10 μm wide) were incubated for 4 h at 37 °C until spread and then their movement was recorded with the use of time lapse video recorder (Panasonic) and CCD COHU high performance camera (Brian Reece Scientific Ltd.).

2.8. Statistical analysis

All results are expressed as means ± 1 s.d. In experiments in which contact guidance of epitenon cells and

BHK cells was studied, area, elongation and orientation of 150–200 cells was calculated for each kind of substratum. For analysis of cell orientation variance was calculated. The unpaired Student's t-test was used to compare groups of results.

To establish whether cell elongation is dependent on groove width and depth, we performed a one-way analysis of variance on the logarithm of the data.

3. Results

3.1. Guidance of epitenon cells on patterned substrata. Comparison with BHK fibroblasts

Cell spread area, elongation and orientation (alignment to the groove direction) was measured in epitenon cells cultured on plain and patterned substratum with varying groove depth and width. This study has been done to establish the sensitivity of epitenon cells to topographical features and find groove parameters that create the best conditions for the guidance of tendon cells. The guidance of epitenon cells was compared to the guidance of BHK cells. Although the two cell lines were obtained from different species, but they represent cell of the same type (fibroblasts) and size (spreading area $2800 \pm 1200\ \mu m^2$).

Epitenon cells were well guided by multiple grooved substrata (Fig. 1). They responded to topographical features by a substantial elongation (Fig. 2). Their elongation did not depend on groove width but showed some dependence on groove depth (one way analysis of variance, $p \ll 0.01$). The best elongation was achieved for 2 and 5 μm deep grooves. Elongation of BHK cells depended both on groove depth and width (Fig. 2). Epitenon cells were significantly better elongated than BHK fibroblasts on shallow grooves 0.5 and 1 μm deep ($p \ll 0.05$).

Epitenon cells were very well oriented on all kinds of gratings, although a decrease in cell orientation was seen for cells grown on shallow grooves, 0.5 μm deep. This is documented by low variance in the tested samples (Fig. 2). BHK cells were well oriented by grooves 2 and 5 μm deep but less oriented by grooves 1 and 0.5 μm deep. Variance for BHK cells was higher than for epitenon cells on all kinds of patterned substrata which shows that epitenon cells are more sensitive to topographical features than BHK fibroblasts (Fig. 2).

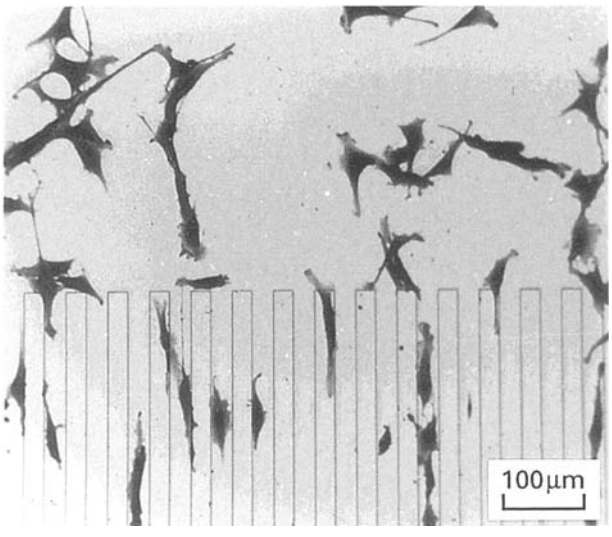

Figure 1 Epitenon fibroblasts on plain (top) and patterned (bottom) substratum. Grooves seen in the lower part of the photograph are 5 μm deep and 25 μm wide. Cells were fixed with 4% formaldehyde and stained with Kenacid blue.

3.2. Tendon healing in organ culture

Tendons were grown for 5 weeks in organ culture on plain and patterned substrata, groove depth 5 μm, width 10 μm. After one week in culture numerous epitenon cells migrated from tendon ends and spread on the substratum. On plain substratum cells were randomly arranged in the space between the two tendon ends (Fig. 3a). On patterned substrata cells migrating from tendon ends were guided by the grooves and formed cellular bridges connecting the two ends of the divided tendon (Fig. 3b). The width of cellular bridge was equal to the width of the tendon since cells were unable to "cross steps" and move across ridges.

After 3 weeks the ends of the tendons grown on plain substratum rounded up and small segments extended towards the gap so that loosely connected thin cellular bridges started to form (Fig. 3c). These delicate bridges were not sufficiently strong to maintain continuity of the tissue when it was handled. In most areas, tendon ends show that rounded up with the formation of a thin cellular layer covering the site of injury (Fig. 4b). Tendons grown on grooves had healed much more completely at this stage and epitenon cells proliferated and migrated into the gap predominantly in places adjacent to grooves (Fig. 4c, d). No alignment of tendon elements was noted at this stage.

At 6 weeks, the tendon was less cellular, the collagen bundles showed marked alignment in the longitudinal axis of the tendon. On Fig. 5b arrows demarcate the sites of tendon division and demonstrate the extent of the new tissue produced by healing. In the newly formed scar tissue the alignment of collagen bundles was seen especially in the area adjacent to the patterned substratum (Fig. 5d). In controls, the accumulation of epitenon cells at the tendon ends was still seen but newly formed tissue was not strong enough to restore the continuity of the divided tendon.

3.3. Epitenon cell movement on plain and patterned substrata

The speed of epitenon cell movement was measured on plain and patterned substrata ($n = 20$). On plain substratum the majority of cells (65%) moved with speed 0–20 μm/h, max speed noticed was 34 μm/h. The mean cell speed was 21.8 ± 8 μm/h (Fig. 6). The mean speed of epitenon cells grown on patterned substrata was not significantly higher than in controls and was 22.6 ± 18 μm/h for 25 μm groove width, 24 ± 17 μm/h for 10 μm groove width and

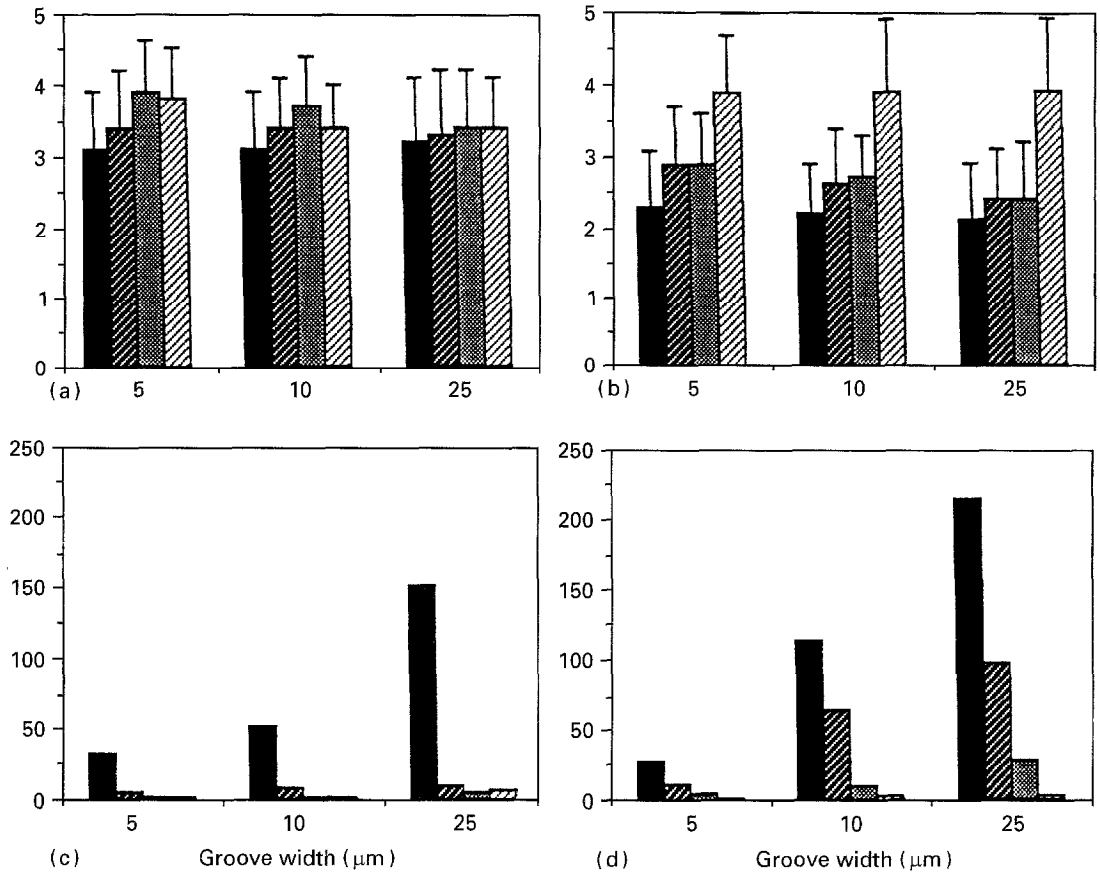

Figure 2 Elongation and orientation of epitenon fibroblasts (a, c) and BHK fibroblasts (b, d) on multiple grooved substrata of groove depth ■ 0.5, ▨1, ▩2 and ▨ μm and width from 5 to 25 μm. Results in cell elongation graphs are means ± s.d. (minimal value of this parameter is 0 for circular objects, and increases as the object becomes more linear). Elongation of epitenon fibroblasts cultured on plain substratum is 1.5 (s.d. 0.9); for BHK cells 1.8 (s.d. 0.8). For cell orientation the angle between groove direction and the long axis of the cell was measured. In this figure changes in cell orientation are shown as changes in the variance of the sample. The variance of control BHK cells grown on plain substratum is 711; the variance of epitenon cells grown on plain substratum is 720.

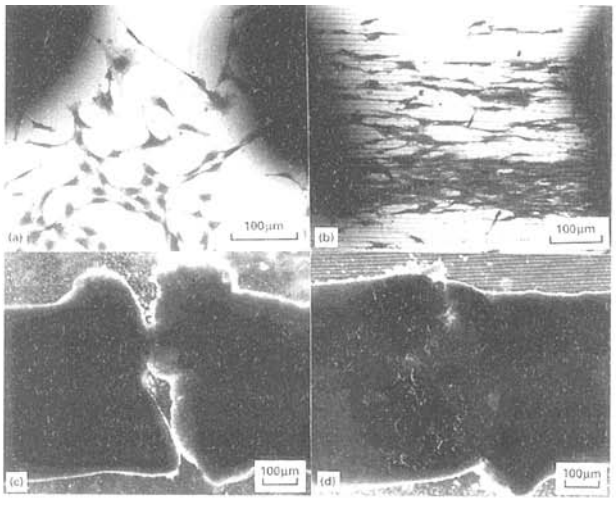

Figure 3 Healing of the rat flexor tendon in organ culture. (a) Epitenon cells randomly arranged between the two ends of the divided tendon, one week in culture. (b) Epitenon cells guided by patterned substratum of groove depth 5 μm and width 10 μm, form "cellular bridges" between the two tendon ends, week in culture. (c) Control tendon on plain substratum, 3 weeks in culture. Tendon on patterned substratum, 3 weeks in culture (d).

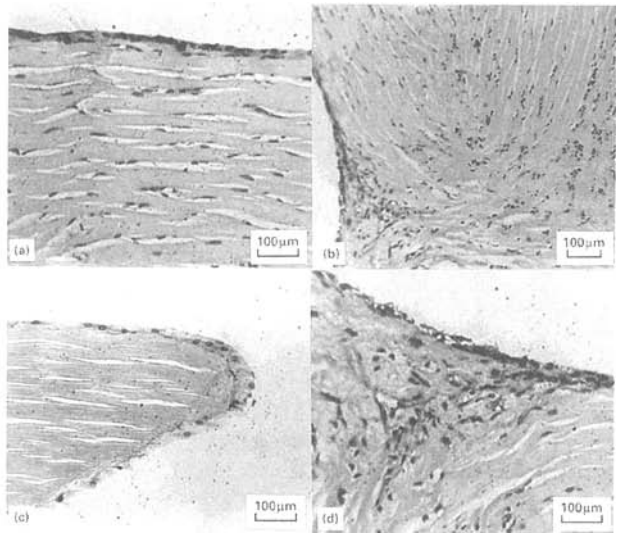

Figure 4 Histological sections of the healing rat flexor tendon after 3 weeks in culture: (a) non-divided tendon; (b) the end of the divided control tendon grown on plain substratum; (c, d) formation of a new tissue in the gap between the two tendon ends in the tendon cultured on patterned substratum. Eosin and haematoxylin staining.

32 ± 30 μm/h for 5 μm groove width (Fig. 6). Although the differences in cell speed on substrata of different groove widths were not significant, the difference patterned–plain substratum was significant ($p < 0.005$). The variance of cell speeds on all kinds of gratings was significantly higher than the variance of cell speeds on plain substratum (50, 16, 17, respectively, for 5, 10, 25 μm grooves versus 4 in controls). This

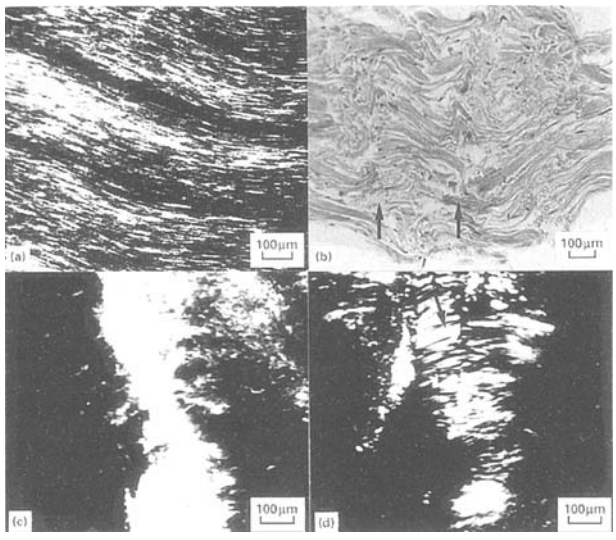

Figure 5 Confocal scanning microscope images of the healing tendon, 6 weeks in culture (a, c, d). (a) Alignment of collagen bundles in control, non-divided tendon, 0.2 μm single plain Z section, reflected light. (c) Upper surface of the divided tendon cultured on patterned substratum. Bright gap between the two tendon ends is still seen. Single plain image, 0.2 μm Z section, reflected light. (d) Alignment of collagen bundles seen in the healing area adjacent to the grooves, 0.2 μm single plain Z section, reflected light. (b) Section of the healing tendon cultured on patterned substratum (area adjacent to the grooved substratum). Alignment of collagen bundles is seen in the space between the two tendon ends demarcated by two arrows. Haematoxylin and eosin staining, bright field.

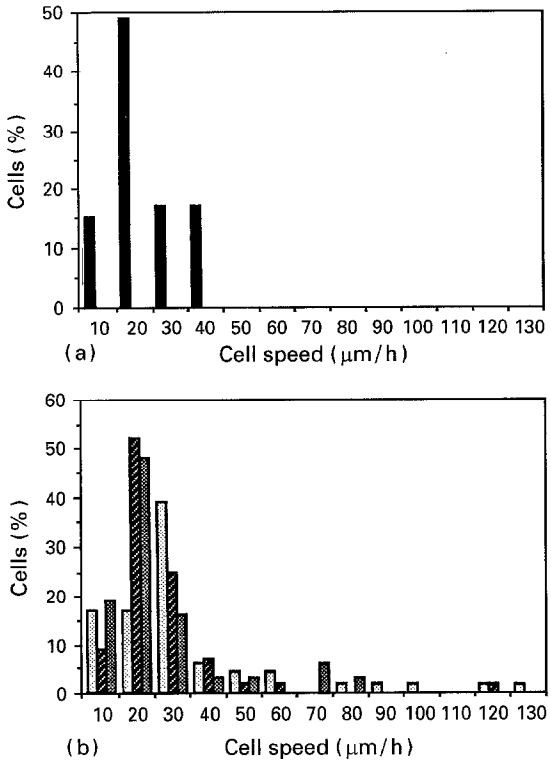

Figure 6 The distribution of speeds in the population of 100 epitenon cells (a) cultured on plain substratum and (b) cultured on patterned substratum of groove depth 5 μm and width ▨5, ▨10, ▨25 μm. The data represent the percentage of cells moving at a certain speed.

means that the diversity of speeds on gratings was much higher than in controls. The range of speeds was 34 μm/h for controls and 150, 116, 76 μm/h, respectively, for 5, 10 and 25 μm wide gratings.

4. Discussion

The use of multiple grooved substrata to produce cell guidance was reported for several cell types [3, 4, 7, 9–12]. Fibroblasts were reported to respond to multiple grooved substrata of certain defined depth and width of grooves by substantial elongation and spreading with the long axis aligning in the groove direction [9, 4]. There have been, however, only a few attempts to use patterned substrata to support physiological processes such as wound healing or to improve biocompatibility of implants [10, 13]. We used multiple grooved substrata to study *in vitro* healing of completely divided rat flexor tendons.

Tendon is a very regular structure where contact guidance seems to play a crucial role in maintaining its function. We report that tendon healing was facilitated by topographical cues as was shown by the analysis of histological sections. Since grooves were found to produce alignment of epitenon cells, their directional migration and increase the speed of cell movement, improved tendon healing may be due to the faster formation of cellular bridges between the two tendon ends. It is also possible that a regular arrangement of epitenon cells promotes regular deposition and maturation of extracellular matrix components. This suggestion is supported by some observations from the clinical practice of tendon repair. It is well established that partial tendon divisions heal with little or no fibrous adhesion formation. The reason for this remains unclear. One possible explanation is that the tension in the tendon produces alignment of newly formed collagen fibres in the long axis of the tendon and acts as a topographical cue for the orderly migration of fibroblasts along the line of the tendon. This possibility is further enhanced by observations of tendon healing following complete tendon division in which the two tendon ends have been sutured. It has been shown that if metal markers are placed at the ends of the divided tendons prior to suture, these markers drift apart very often by as much as 1 cm between the time of injury and the end of healing process. This phenomenon known as "tendon gapping" suggests that the muscle tension stretches the fibres out and may provide guidance cues in the same way as it was observed in cells cultured on stretched plasma clots [14–16]. Tendons which heal with significant gapping show no increased tendency to adhesion formation compared to tendons which heal by primary union. It might be expected that they would behave otherwise and increase the risk of haphazard migration of tenocytes within the synovial space.

It might be possible to design a membrane which could be wrapped around the tendon at the time of repair. If this membrane had appropriate grooves in its inner aspect it could promote orientation and migration of tenocytes to heal the tendon. If the outer surface of the sheath was non-adherent to cells it might help to form a new sheath around the repairing tendon. In this respect it has been known for some time that silastic rods implanted into tendon sheaths following excision of severely damaged and adherent tendons promote the formation of a false sheath round

about them and that when a subsequent tendon graft is inserted into the tunnel which the silastic has provided, adhesion formation does not take place.

In conclusion, multiple grooved substrata facilitated *in vitro* healing of completely divided flexor tendons. This effect may be caused by faster formation of cellular bridges between the two tendon ends. Cells isolated from the surface of the tendon were reported to align in very shallow grooves and move with higher speed than cells cultured on plain substrata. These observations may find several applications in the clinical practice of tendon repair.

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