JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **12** (2001) 1005–1011

A study of the cellular response to orientated fibronectin material in healing extensor rat tendon

F. ZAVAHIR, D. A. McGROUTHER, A. MISRA, K. SMITH, R. A. BROWN, V. MUDERA*
University College London, RFUCMS, Centre for Plastic and Reconstructive Surgery, Tissue Repair Unit, 67–73 Riding House Street, London W1W 7EJ, UK
Email: v.mudera@ucl.ac.uk

3D orientated fibronectin (Fn) mats have been used as biocompatible and biodegradeable scaffolds to provide orientated cues using contact guidance for cell migration/adhesion and deposition of extracellular matrix. We have implanted Fn scaffolds in an established rat tendon(partial tenotomy) injury model to test its efficacy and monitor the early cellular and inflammatory response. Tendons were harvested at 0, 6 h, 1, 3, 5, 7 and 14 days for H&E, immunohistochemistry and TEM. Total cell counts within the window increased progressively with time with no significant differences between the Fn scaffolds and controls. CD45 (pan leukocyte) positive cell numbers peaked at 6 h and when expressed as a percentage of total cell counts as determined by H&E staining constituted 20% of the total cell number at 6 h but decreased to 5% of total number by 72 h. There were no significant differences in the inflammatory response between the control and implanted groups. Few CD44 (mesenchymal stem cell) positive cells identified had a surface location. A novel cell with long exaggerated cytoplasmic processes was identified by TEM. Our results show that the Fn scaffold did not degrade or elicit any untoward inflammatory response at the time points tested and has potential use in guiding the repair process.
© *2001 Kluwer Academic Publishers*

Introduction

Tissue engineering is an interdisciplinary field that incorporates the principles of engineering and life sciences, to improve and restore tissue [1]. One example of this has been to bioengineer an orientated matrix scaffold, which would mimic the normal *in vivo* matrix, which with engineering modifications would provide cell guidance cues and required orientation to restore tissue micro architecture. This template might encourage host cells to deposit new fibrous tissue in a desirable manner. One primary application would be surgical implantation of such a scaffold into an area requiring tissue replacement and reorganization, during the healing process.

Fibronectin (Fn) is known to play a critical role in wound healing, supporting adhesion, proliferation and motility of cells migrating into the wound bed [2]. Soluble Fn is present as a plasma protein and insoluble fibers of Fn are found in early stage extracellular matrix following injury. Techniques for forming orientated biodegradable 3D Fn mat implants [3] have been developed to make longitudinally aligned cell attachment matrices and have been shown to promote the directional repair of peripheral nerves in rats [4]. We have tested the effects of these Fn scaffolds to provide orientated contact guidance for cell migration/adhesion [5], extracellular matrix deposition and integration of wound edges to potentially improve tendon repair.

The initial stage in the study of the effects of orientated fibronectin scaffolds implanted into tendons, has involved comparing early localized cellular responses in an established tendon window injury model with and without the orientated Fn scaffold. Assessing the immunological response to the implanted biomaterial in tendons would form the basis for developing this bioartificial tissue construct for therapeutic application to tendon and ligament healing.

Materials and methods

Bioengineered orientated fibronectin mats were prepared as described by Brown *et al*. [6]. Briefly, plasma fibronectin solution was concentrated in an ultrafiltration cell (Amicon PM10 membrane; Millipore, Watford, UK) pressurized with nitrogen under continuous stirring at 4 °C. This deposited an orientated fibrous mat of fibronectin by shear aggregation on to the shaft of the stirrer which was rinsed in distilled water, freeze dried and sterilized by gamma irradiation prior to implantation.

Sprague-Dawley rats weighing 200–250 g were used. The partial tenotomy window injury model of Mathew and Moore [7] was used in which a full thickness window

* Author to whom correspondence should be addressed.

0.5 mm wide, 5 mm long was made in the extensor digitorum longus tendon. Briefly, the dorsum of the hind paw was shaved and a V-shaped incision made. The base of the flap was placed proximally and reflected to expose the paratendinous tissue. A total of four tendons per hind paw were dissected out using micro scissors.

Orientated fibronectin (Fn) mats cut to fit this window with their fibers aligned parallel to the tendon and held in place by a 10/0 Ethilon suture placed in the middle. Control tendons did not receive any fibronectin mats. The skin flap was sutured with 8/0 Nylon suture and the rats allowed to recover. Animals were sacrificed as per Home Office Regulations and tendons harvested at 0, 6 h, 1, 3, 5, 7 and 14 days post operation for histology, immunostaining and transmission electron microscopy (TEM).

Harvested tendons at 0, 6, 24 and 72 h were fixed in neutral buffered formaldehyde, processed routinely and wax embedded. Ten micron sections were cut for staining from 48 tendons (12 per time point, 6 control, 6 Fn mat). Cell nuclei were counted in each case in the window and surrounding tendon (Fig. 1), using a 40 × lens on a light microscope (Leitz, Portugal). Counts were made per calibrated graticule field (600 μm × 500 μm) and covered the whole tendon section. Determinations were an average of at least 2 counts per section with cell counts grouped into different regions of the tendon (Fig. 1).

Forty eight tendon sections (6 control and 6 Fn mat) at 0, 6, 24 and 72 h were snap frozen in liquid nitrogen and stored at $-80\,^{\circ}$C for immunostaining. CD 45, a pan leukocyte surface antigen [8], was used to identify inflammatory cells cryosections from these specimens. Sections on Vectabond coated microscope slides were fixed in methanol for 10 min, washed for 5 min in PBS three times, blocked with 1% bovine serum albumin in PBS for 20 min in a humidified chamber at room temperature and blot dried. Sections were treated with 20 μl of anti-CD45 (1/100 dilution in PBS (mouse anti-rat monoclonal IgG1, Serotec UK) incubated for 30 min at 37 °C, washed three times in PBS as above, further incubated at 37 °C with 20 μl of polyclonal goat anti-mouse conjugated to FITC 1/10 (30 min), washed in PBS, mounted in Aquamount and viewed on a 50 × lens Leitz, Portugal microscope. Rat white cell buffy coat smeared slides served as positive controls and the primary antibody omitted for negative controls.

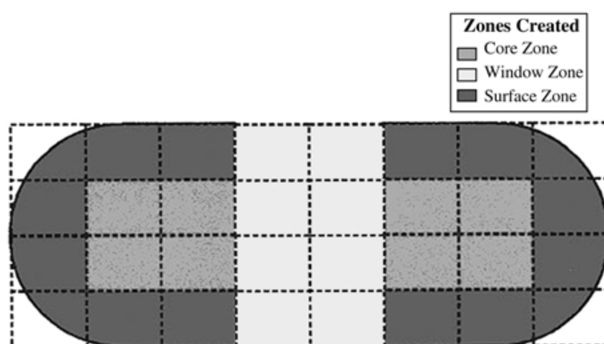

Figure 1 Diagram of the graticule grid used to differentiate the transverse tendon sections into surface, core and window zones for cell counts.

Immunostaining for CD 44 a surface antigen described for mesenchymal stem [9] used 15 micron cryosections as described above but blocked using 20% goat serum (diluted in PBS) for 20 min. Sections were treated with neat anti-CD 44 for 30 min (mouse anti-rat, monoclonal antibody; 37 °C), washed in PBS, incubated at 37 °C with a polyclonal FITC goat anti-mouse IgG conjugated at a dilution of 1/10, for 30 min followed by three PBS washes and mounted and viewed as before. Rat marrow smears served as positive controls and the primary antibody omitted for negative controls.

In total 28 tendons were processed for transmission electron microscopy (TEM). The tendons were harvested at 6 ($n=3$), 24 ($n=3$), 72 ($n=3$) hours, 5 ($n=7$), 7 ($n=8$) and 14 ($n=4$) days post injury and fixed in 1.5% gluteraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The tissues were post fixed in 1% osmium tetroxide (Agar Scientific, Stansted, Essex, UK) in 0.1 M phosphate buffer. Specimens were dehydrated, transferred to propylene oxide for embedding in Spurrs epoxy resin (Agar) under vacuum infiltration and polymerized at 70 °C for 18 h. Transverse sections were cut through the tendon and injury site. 1 μm sections were stained with toluidine blue for light microscopy. Ultra-thin sections were stained with uranyl acetate and lead citrate.

Cells counts were expressed as mean ± standard error of the mean (SEM). Paired Student's *t*-test was used to compare differences between the Fn mats implanted tendons and untreated tendons, $p < 0.05$ was considered to be significant.

Results

No cells were seen within the surgically created window at 0 h in either the implanted or non-implanted tendon as would be expected. By routine histology, injury margins and intact tendon showed the elongate nuclei of resident fibroblasts (Fig. 3a). The uninjured tendon core was sparsely populated and the tendon surface had a ring layer of 1–2 cells consistent with normal tendon structure. By 6 h post surgical injury, the total cell number in the non-implanted window injury site was 36.3 ± 6.5. The fibronectin implanted window total cell number at 29.3 ± 6.2, was not statistical difference and is consistent with a normal cellular response to injury. Mean cell count did not increase significantly by 24 h (Fig. 2a) but by 72 h this has risen significantly for non-implanted and mat implanted tendons (170.8 ± 72.4 and 123.0 ± 13.0 respectively, $p < 0.05$). At no stage was there any statistical difference between implanted and non-implanted specimens.

Fig. 2b shows the profile of core cells over 72 h. The cell numbers did not change significantly in the first 6 h in the mat implanted and non-implanted specimens. At 24 h, core mean cell counts in the non-implanted tendons were 48.5 ± 13.2 and 31.2 ± 4.2 in the mat implanted tendons. Over the time points tested up to 72 h, tendon core cell counts did not change significantly either between time points or between mat implanted and non-implanted tendons.

The surface of a normal uninjured tendon shows surface fibroblasts forming a thin 1–2 cell layer outer

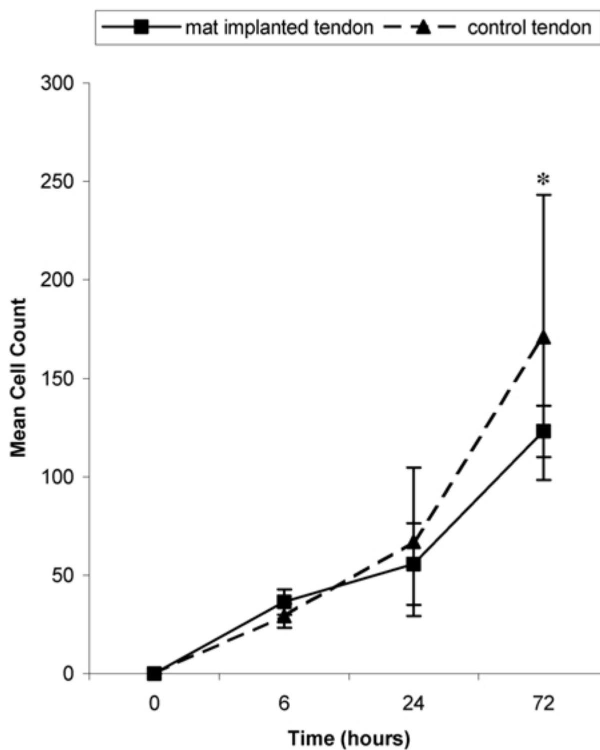

Figure 2a Graph of total mean cell count in the injury zone of the tendon (window) vs. time. Note the increase in mean cell count with time upto 72 h tested. There is no statistically significant difference between control and Fn mat implanted tendons over time. However, total mean cell counts increased significantly for both groups by 72 h when compared with 6 h. *Indicates $p < 0.05$.

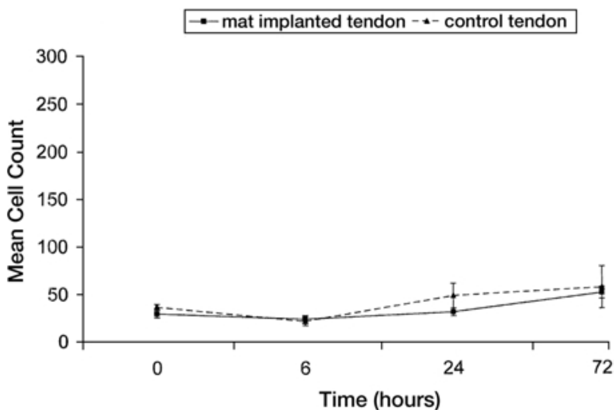

Figure 2b Graph of mean total cell count in the tendon core vs time for Fn implanted and control tendons. *Note:* The mean total cell count did not change significantly between the control and the Fn mat implanted tendons at the time points tested.

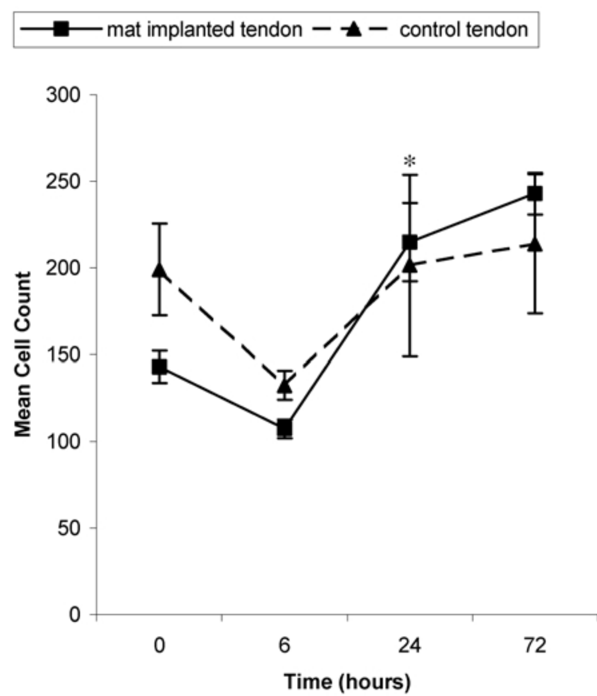

Figure 2c Graph of mean total cell count in the surface of tendon vs. time. *Note:* The mean surface total cell counts for non-implanted and fibronectin mat implanted tendons at 6 h were 34% and 25% lower compared to 0 h which increased significantly by 24 h (52% and 100%). This further increased by 13% and 6% in the mat implanted control tendons by 72 h though this rise was not significant. $p < 0.05$.

coat, with densely packed cells when compared to the core. The mean surface cell counts for non-implanted and fibronectin mat implanted tendons were at 6 h 34% and 25% lower respectively compared to 0 h (Fig. 2c), though this did not reach statistical significance ($p > 0.05$). There were increases in the surface mean cell counts at 24 h in the non-implanted tendons; 52% and 100% in the mat implanted tendons. This level of cellularity did not change upto 72 h. The surface counts reached to their highest level at 72 h in both groups; 13% in the mat implanted tendons and 6% in the control tendons compared to the previous 24 h time point. The increase in cell number (6–24 h) was mirrored by an increase in number of cell layers in the surface of the tendon from 1 to 2 cells deep at 0 h (Fig. 3a) up to 10–11 layers at 72 h near the surgically created window (Figure 3b), consistent with the normal cellular response to tendon injury as described earlier by Gelberman *et al.* [10] and Mass *et al.* [11]. Importantly, no difference was seen with the Fn implant.

The CD 45 inflammatory cells were counted in all the zones of the tendon (0, 6, 24 and 72 h). No inflammatory cells were found in the tendon core and surface zones of the tendon for any of the time points tested. However positively stained cells were visualized in the window zones in both control and Fn mat implanted tendons. The maximum rate of increase in CD 45 inflammatory cells in the window zone was seen in the first 6 h (Fig. 4a), indicating rapid maximal inflammatory response though this was unaffected by the Fn mat. At 6 h the mean inflammatory cell count in the window region for the Fn mat implanted tendon per section was 6.7 ± 1.1 and in control tendons per section 4.8 ± 0.7 (Fig. 4a). There was no significant differences in the total inflammatory cell counts between the two groups and after 6 h (to 72 h) the mean cell count remained the same. When expressed as a percentage of total cell counts as determined by H&E staining CD45 positive cells constituted 18% of the total cell number at 6 h, 8% at 24 h and 4% of total number by 72 h in the Fn mat implanted windows with no significant differences between the control and implanted groups (Fig. 4b).

CD44 immunostaining in both groups did not show any positive stained cells at 0 or 6 h. At 24 h 1–2 positively stained cells were identified per tendon section. These stained cells were found exclusively in the tendon surface zone and never in tendon core. Again

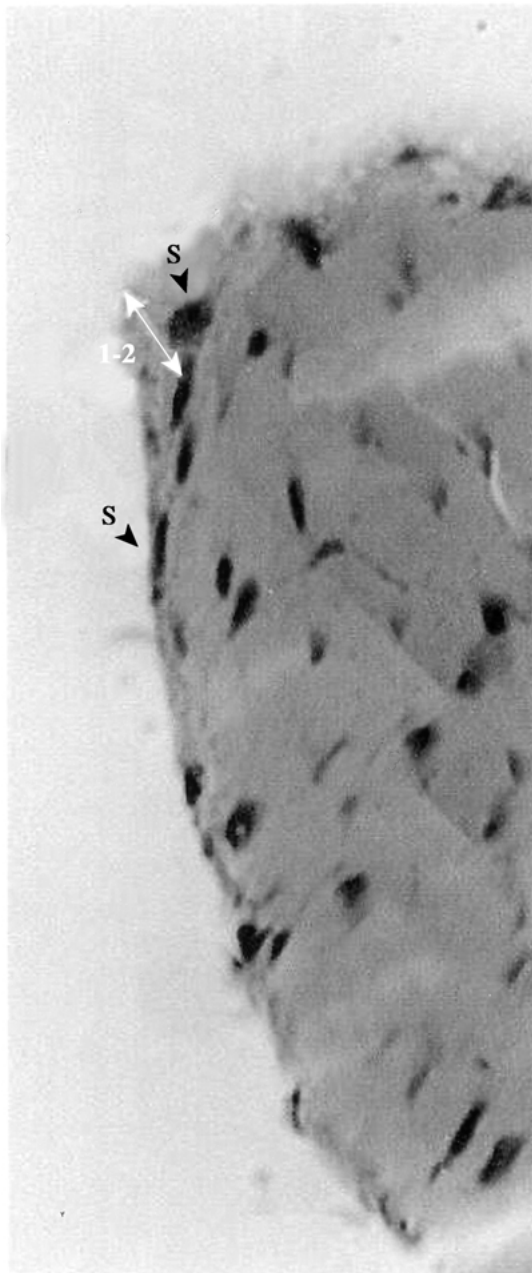

Figure 3a H&E stained transverse section of the surface layer of tendon showing elongated stained nuclei at 0 h. 40×. *Note:* (S) Surface of tendon showing 1–2 cell layer representing normal morphology.

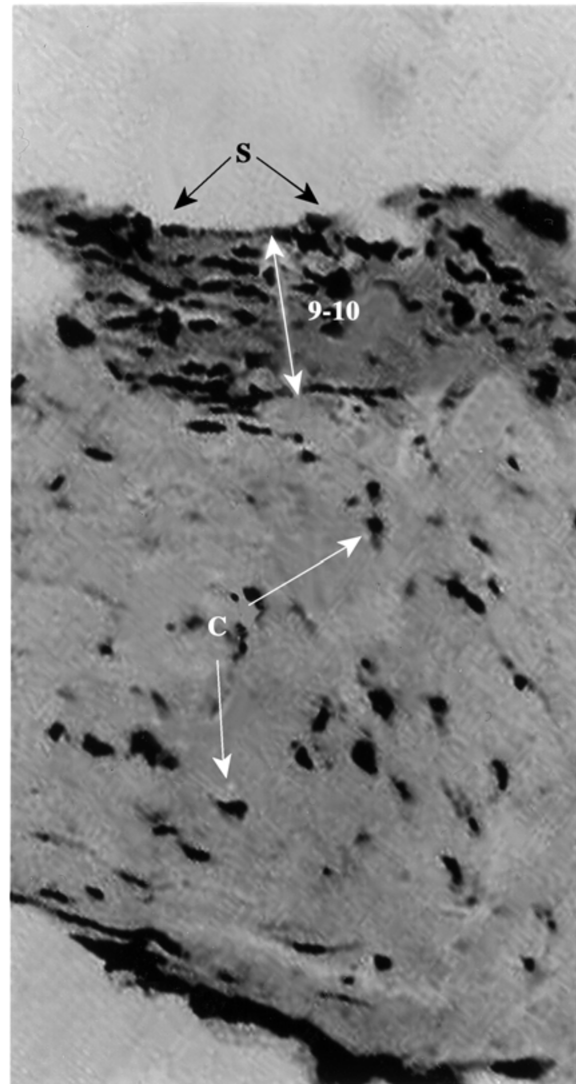

Figure 3b H&E stained transverse section of the surface layer of tendon showing elongated stained nuclei at 72 h. 40×. *Note:* (S) Surface layers have increased from 1–2 cells to 9–10 cells. (C) Sparsely populated core cells which remained unchanged throughout the time points studied.

at 72 h, 1–2 cells per section were also CD 44 positive, again exclusively found in the surface of the tendon. CD 44 positive cells were too few for detailed analysis though they seemed to appear as part of the rapid increase in cell number seen after 6 h as seen in H&E cell counts. As before, there were no differences in the distribution of CD 44 positive cells between treated and control wounds upto the 72 h stage.

Ultrastructural analysis was used to assess the morphology of the cellular infiltrate in the tendon window and wound margins. The cells identified morphologically were grouped into fibroblasts, neutrophils, all other inflammatory cells (including lymphocytes). A novel cell with long exaggerated cytoplasmic processes was identified in the window region and wound margins in the Fn implant and control tendons. It was observed to engulf large chain collagen fibrils (Figs 5a and b), separating them, as if to facilitate the laying down of fresh collagen and aid integration. A few random novel cells were morphologically identified as early as 6, 24 and 72 h post injury in the Fn mat implanted tendons and control tendons. A greater number of these cells were identified embedded in the mat implants between five and 14 days.

Discussion

Orientated fibronectin mats have been shown to support peripheral nerve regeneration in a rat sciatic nerve model, with significantly greater axonal regrowth compared to freeze-thawed muscle graft controls [4]. This study also showed accumulation of highly aligned new supporting collagen around the regenerating nerve. This raised possibility of engineering tendon healing using similar fibronectin scaffolds to guide orientated connective tissue repair. Using the same window injury model in Fisher rats without any implant Iwuagwu *et al.* [12] showed mainly inflammatory cells in the window at 6 h. These cells constituted the main cell population in the window zone up to 24 h. Our study again showed a

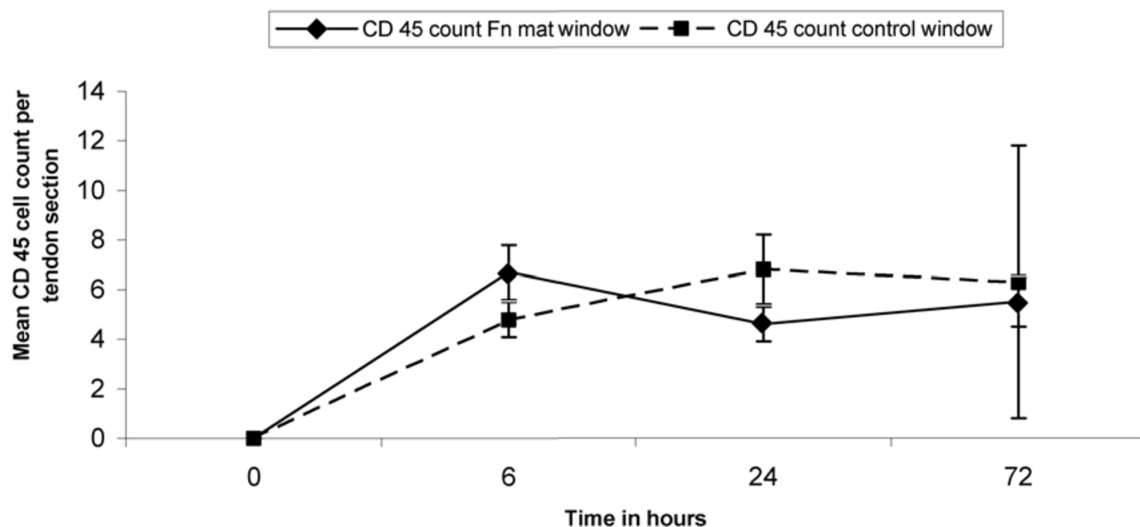

Figure 4a CD 45 (pan leukocyte) positive cell counts in Fn mat implanted tendon and control tendon vs. time. *Note:* The maximum rate of positively stained cell increase (inflammatory response) in the window zone was seen in the first 6 h with no significant difference between the two groups. The counts plateaux off at 24 h.

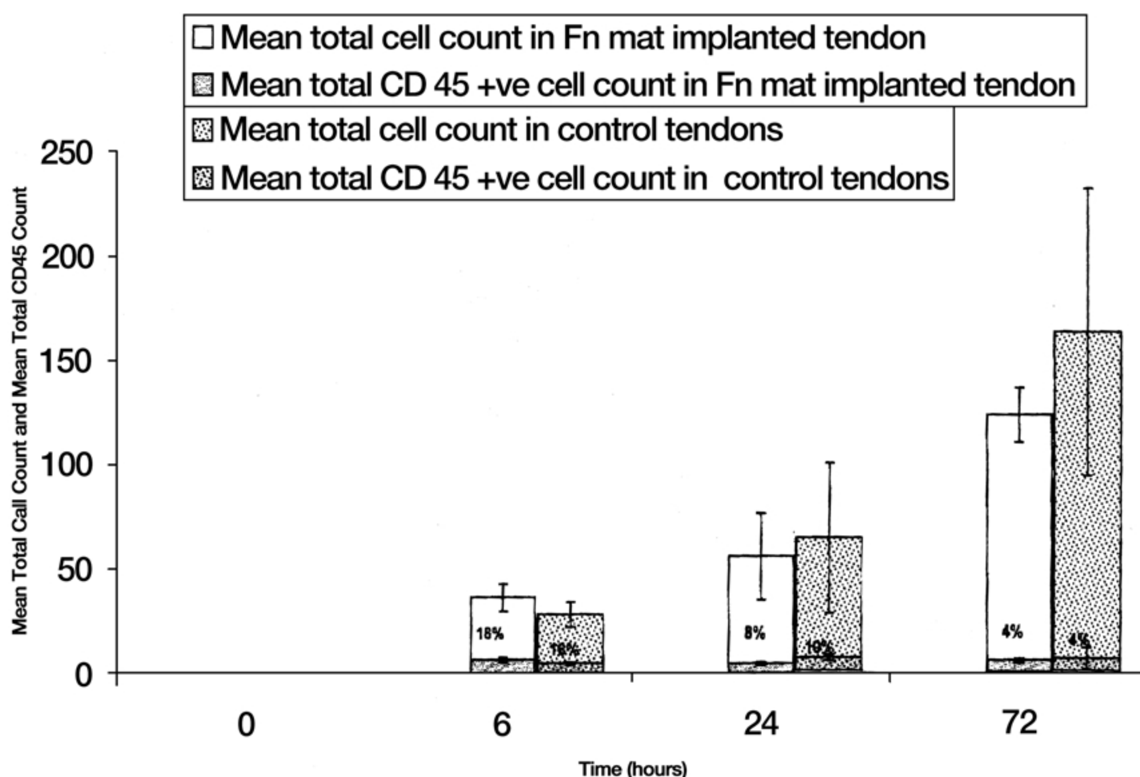

Figure 4b Stacked bar diagram of the mean total cell count with % CD45 positive cell count vs. time. The inflammatory cell count is expressed as a percentage of mean total cell counts. *Note:* The Fn mats did not elicit any untoward inflammatory response compared to control injury.

peak inflammatory response by 6 h in the non-implanted tendons. Significantly, the Fn mat implanted animals did not show any untoward inflammatory response at any of the time points and though peaking at 6 h, inflammatory cells only comprised 18% in Fn mat implanted tendon compared with 16% in non implanted tendons. Our findings are consistent with previous reports that maximal inflammatory response is elicited by 6 h [13].

Cell attachment to a substratum is an essential part of migration. An ideal matrix for this is fibronectin fibers [14]. Studies of the localization of fibronectin in surface tendon cells, during healing in flexor tendons [15, 16], indicated a peak accumulation in canine tendon surface cells, at day 7, indicating that onset of surface cell migration is not an immediate process probably following peak inflammatory cell infiltration.

Tendon core cell counts remained unchanged throughout the 72 h studied. This suggests a possible resistance of core cells to early environmental cues and triggers. Tendon core cells in a rabbit tendon healing model have been shown to apoptose [17] using a p53 protein assay by day 7 around the injury zone. Window tenotomy alters tension in the collagen matrix surrounding the window injury and it has been reported that cells that lose the resting tension imparted by the matrix die (anoikis) by apoptotic mechanisms [18].

Gelberman *et al*. [10] using forelimb flexor tendons in partially immobilized dogs (post operatively) showed

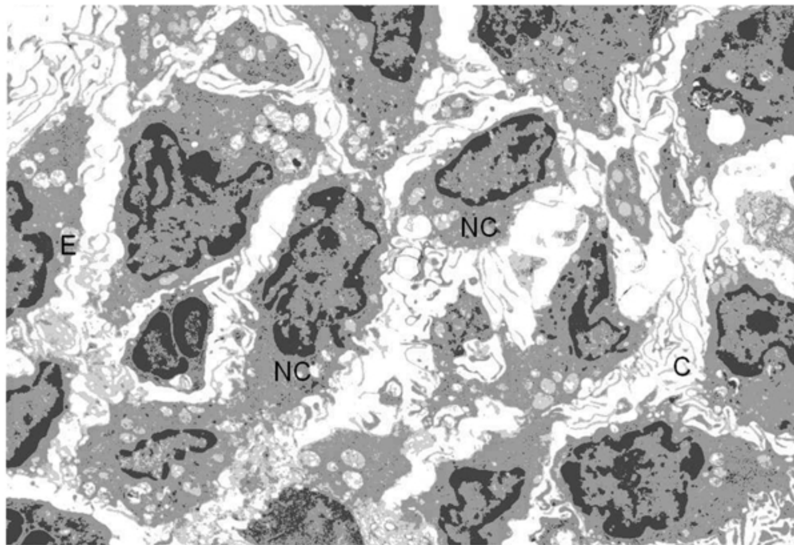

Figure 5a Transmission electron microscopy picture from a rat extensor tendon window region with the fibronectin mat insitu (7 day time point). *Note:* The novel cells (NC) with their long cytoplasmic projections (C). Magnification 5.5 K.

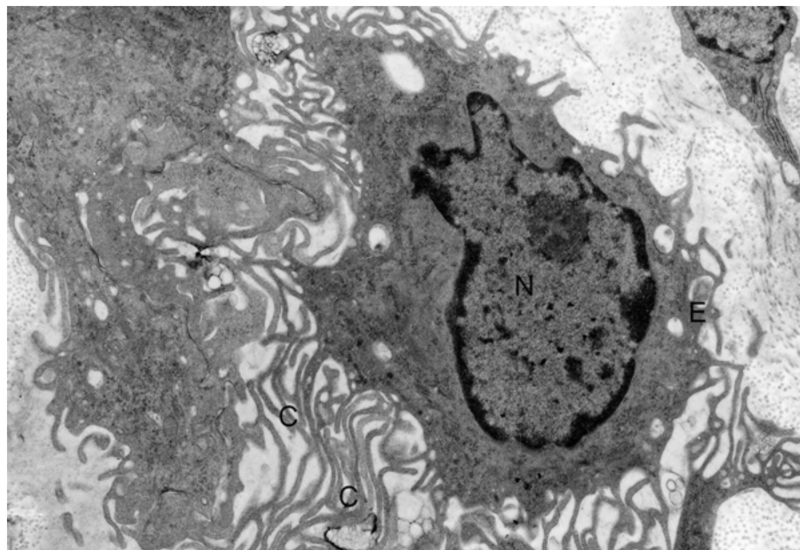

Figure 5b Transmission electron microscopy picture of the novel cell at a higher magnification (9.6 K). Note the long slender cytoplasmic projections (C), large nucleus (N) and the cytoplasmic projections seem to engulf (E) tissue.

that surface cells (epitenon) increased by day three to five cell layers. These cells continued to increase to 15–20 cell layers by day 17. In another study [11] human hand flexor tendons harvested from amputated arms were placed in culture medium after surgical injury. The surface cells increase to 10 cell layers though this took 8 weeks. These observations are consistent with our study where the surface cell layers increased to 9–11 layers 72 h post injury. Similar multiplication of cell layer thickness was achieved in 72 h in our study, and in Gelberman's canine model and eight weeks in Mass and Tuel's *in vitro* human explant culture. This might be due to the different tissue injury models used. Flexor tendon is subjected to greater loading *in vivo* in comparison to extensors [19]. Another possibility might be the different models employed by the various investigators. Abrahamsson [20] suggested the rodent has the most rapid rate of *in vitro* tendon healing, and reported *in vitro* tendon healing to be much slower and more ''dis-organized'' than *in vivo* models.

Presence of resident stem cells in tissues has been documented in tissues where there is high cells turnover and cells must be continually replaced [21]. Stem cells have been identified in the skin, intestine and hemato-poietic systems. The presence of stem cells in tissues with low cell turnover has also been documented, for instance, the satellite cells in skeletal muscle [22] and oval cells in the liver [23]. In our study, rat tendons did not stain for mesenchymal stem cells (MSC) marker CD 44 until 24 h. The cells that did stain were very low in number but were exclusively found on the surface of the tendon. As no staining was seen at 0 h, the suggestion is that MSCs may not be resident in the tendon. If so from where do they originate? Infiltration of MSCs from the circulation would be expected to lead to greatest accumulation in the window injury with other circulating blood cells. Exclusive localization on the surface on the contrary, is more consistent with the origin being the either resident tendon or originating and migrating from the surrounding connective tissue fascia.

Stem cell morphology has been described in the past [24]. The study using time-lapse acquisition photography to study a hematopoietic progenitor cell line found numerous thin and thick cytoplasmic extensions, protruding and retracting from the cells examined. The novel cell identified only morphologically at ultrastructural level in our study (common at this site between five and 14 days post injury) also possesses extensive, long slender cytoplasmic projections. Initial studies suggests that these cells seem to play an important role in the integration of the healing tendon, however, it is likely to play a role at later time points and this is currently under detailed investigation. Fibronectin is a known substrate for stem cells [24]. The role of Fn mat implants in progressive accumulation of such cells and fibrous repair is the subject of our ongoing studies. However, these are likely to be later stage events (> 3–5 days post injury). Our present work clearly illustrates that the Fn mat implants elicited no significant inflammatory response from normal nor have any deleterious effects on the initial repair process.

Acknowledgment
We are grateful to Action Research for their support.

References
1. R. LANGER and J. P. VACANTI, *Science* **280** (1993) 920–926
2. R. A. F. CLARK, J. M. LANIGAN and P. DELLAPELLE, *Invest. Dermatol.* **79** (1982) 264–269.
3. O. S. EJIM, G. W. BLUNN and R. A. BROWN, *Biomaterials* **14** (1993) 743–748.
4. I. H. WHITWORTH, R. A. BROWN and C. DORE, *J. Hand Surg.* **4** (1995) 429–436.
5. Z. A. AHMED and R. A. BROWN, *Cell Motil. Cytoskel.* **42** (1999) 331–343.
6. R. A. BROWN, G. W. BLUNN and O. S. EJIM, *Biomaterials* **15**(6) (1994)
7. C. A. MATTHEW, M. J. MOORE and L. CAMPBELL, *J. Hand Surg.* **12B**(3) (1997), 313–320.
8. I. ROITT, J. BROSTOFF and D. MALE, in ''Immunology'' (Mosby International Ltd, London, 1998).
9. M. F. PETTINGER, A. M. MACKAY, S. C. BECK, R. K. JAISWAL and R. DOUGLAS, *Science* **284** (1999) 143–147.
10. R. H. GELBERMAN, J. S. VANDEBURG, P. R. MANSKE and W. H. AKESON, *J. Hand Surg.* **10A** (1985) 776–784.
11. D. P. MASS and R. J. TUEL, *J. Hand Surg.* **16A** (1991) 24–31.
12. F. C. IWUAGWU and D. A. MCGROUTHER, *Plastic and Reconst. Surg.* **102**(6) (1998) 2064–2071.
13. R. A. F. CLARK, in ''Molecular and Cell Biology of Wound Repair'' Second edition (Plenum Press, New York, 1993) 3–50.
14. B. WOJCIAK-STOTHARD, M. DENYER, M. MISHRA and R. A. BROWN, *In Vitro Cell Dev. Biol.* **33** (1997) 110–117.
15. D. AMIEL, R. GELBERMAN, F. HARWOOD and D. SIEGEL, *Matrix.* **11** (1991) 184–189.
16. R. H. GELBERMAN, D. STEINBERG, D. AMIEL and W. AKESIN, *J. Hand Surg.* **16A**(4) (1991) 686–693.
17. S. KAKAR, U. KHAN and D. A. MCGROUTHER, *J. Hand Surg.* **23B**(5) (1998) 627–632.
18. S. M. FRISH and H. FRANCIS, *J. Cell Biol.* **124**(4) (1994) 619–626.
19. R. A. ELLIOTT JR., in ''Plastic Surgery'' (WB Saunders and Company, New York, 1990) 4567.
20. S. O. ABRAHAMSSON, *Scand. J. Plast. Reconstr. Surg.* **23**(Suppl.) (1991) 1–51.
21. C. R. R. BJORNSON, R. L. RIETZA, B. A. REYNOLDS, M. C. MAGII and A. L. VASCOVI, *Science* **283** (1999) 534–537.
22. P. SEALE and M. A. RUDWICK, *Dev. Biol.* **218** (2000) 115–124.
23. B. E. PETERSON, W. C. BOWAN, K. D. PATRANE, W. M. MARS, A. K. SULLIVAN, N. MURANE, S. S. BRIGGS and J. S. GREENBURGER, *Science* **284** (1999) 1168–1170.
24. K. FRANCIS, R. RAMAKIRISHNA, W. HOLLOWAY and PALSSON, *Blood* **92**(10) 3616–3623.

Received 14 May
and accepted 21 May 2001