Modulation of the formation of adhesions during the healing of injured tendons

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The formation of restrictive adhesions around the musculotendinous unit after injury is one of the most vexing processes faced by the surgeon. In flexor tendons it has been shown that the synovial tissue is the source of aggressive fibroblasts which contribute to this process. Using a rabbit model, we have examined the effects of treating the synovial sheath with the antimetabolite 5-fluorouracil (5-FU) for five minutes. Inflammatory, proliferative, and molecular markers were compared in the response of the treated and control tendons to injury. Compared with a control group we found that the proliferative and inflammatory responses were significantly reduced (p < 0.001) in the treated tendons. Not only was there a reduction in the cellular and cytokine response, but there also was a significant (p < 0.001) reduction in the level of activity of the known pro-scarring agent, transforming growth factor beta 1 (TGF-β1). These pilot studies indicate that the formation of restrictive adhesions may be modulated using a simple single-touch technique in the hope of producing a better return of function.

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Adhesions form at various sites throughout the body and cause morbidity. At times they occur regularly but their nature and extent are unpredictable. After division of the long flexor tendons of the hand, accurate surgical repair is often coupled with a dedicated rehabilitation programme, which may improve the functional outcome. The patients, often young, may find it impossible to return to work. Efforts have been made to try to elucidate the cellular and molecular mechanisms which lead to the formation of adhesive tissue. Methods to reduce this in injured flexor tendons have included the use of anti-inflammatory agents, hyaluronan, electric fields and ultrasound. It has been shown that after injury the synovial sheath reacts with a greater proliferative and inflammatory response compared with the endotenon. Recent attention has focused on the populations of fibroblasts which are available to the injured site. The synovial fibroblasts are more reactive to growth factors and have a greater capacity for degradation of the matrix. The question arises as to which aspect of cell behaviour should be influenced in order to reduce the formation of adhesions. Monoclonal antibodies are available which decrease the action of transforming growth factor beta-1 (TGF-β1) in cutaneous tissue to reduce the formation of scar tissue. Inhibitors of the matrix metalloproteases have also been shown to lessen the contracture of scar tissue. These refined methods of controlling cell behaviour may prove to be expensive and hence have limited application. Antimetabolites such as mitomycin-C and 5-fluorouracil (5-FU) have been used extensively by ophthalmic surgeons to control the formation of scar tissue in the injured eye. The use of the ‘single-touch’ technique as a possibility of significantly reducing the behaviour of synovial fibroblasts and formation of adhesions in the tendon unit is appealing, but how 5-FU produces its effects is unknown. We have therefore applied the single touch of 5-FU to the injured synovial sheath of rabbits after partial laceration of the tendon. We analysed the tissue using electron microscopy and immunohistochemistry to evaluate those aspects of cell behaviour which are important in the formation of adhesions to elucidate whether they were influenced by the treatment with 5-FU.

Materials and Methods

We operated on adult male New Zealand white rabbits, weighing 2.8 to 3.3 kg, after they had acclimatised to their new environment for one week. The digits of the right hindpaw were used separately in 15 rabbits in a total of 60 tendon operations, split equally between the treatment and control groups using a dual-pair random design so that
there were two control and two treated tendons. The experimental conditions conformed to the British Home Office regulations. The rabbits did not act as their own controls.

**Adhesion model.** In an initial study the combination of tendon and sheath injuries with adequate immobilisation was found to be a potent stimulus for the consistent formation of adhesions. A partial tendon injury was made in which about half the diameter of the flexor digitorum profundus (FDP) was incised in a standard fashion between the A2 and A3 pulleys after emerging from the bifurcation of the flexor digitorum superficialis (FDS). An immobilising ‘stay’ suture was placed proximal to the site of injury.

**Operative procedure.** Each rabbit was weighed and premedicated consisting of intramuscular Hypnorm (0.3 ml/kg) and Hypnovel (0.2 ml/kg) (Sigma, Poole, UK) was given. A single dose of enrofloxacin (0.3 ml/kg) was given as a prophylactic antibiotic after shaving the right hind paw. Anaesthesia was maintained with 0.5% halothane and oxygen at a flow rate of 2 l/min through a gas mask. Vetersgesic (0.3 ml/kg) was given after operation for analgesia. Surgery was undertaken under aseptic conditions aided by an operating microscope (Carl Zeiss, Hannover, Germany). The right hind limb of each animal was prepared with 0.5% chlorhexidine solution and draped after which a tourniquet was applied above the os calcis.

We made separate longitudinal midline skin incisions on the volar aspect of the proximal phalanx of each digit. Careful dissection was carried out to expose the synovial sheath. This was incised transversely between the A2 and A3 pulleys to gain access to the FDP distal to the bifurcation of FDS. An ophthalmic cellulose sponge (Altomed, Gateshead, UK) was delivered on to the sponge using a needle and syringe. By gently applying intermittent pressure, the solution of 5-FU was encouraged to spread throughout the synovial space via the sponge which was changed twice during the application time of five minutes. A mean volume of 0.4 ml of 5-FU was used per animal. The sponge was then removed and the area irrigated thoroughly with phosphate-buffered normal saline (PBS). The two control tendons were treated by irrigation with PBS.

Standard tendon injuries were then made by lifting the FDP with a curved microforceps just distal to its emergence from the bifurcation of the FDS and incising through half of its substance adjacent to the point of injury to the synovial sheath. This method of lifting the tendon ensured that there was minimal tissue handling so that the variability in experimental conditions was reduced. An immobilising 6/0 nylon ‘stay suture’ was inserted at the level of the metacarpoophalangeal (MCP) joint through the transverse metacarpal ligament into the tendon-sheath complex to immobilise both tendons and the synovial sheath as a single unit and to encourage the formation of adhesions. The tendon and sheath incisions were not repaired. Skin closure was with 6/0 nylon interrupted stitches. A firm Expandover bandage (Sherwood Medical, St Louis, Missouri) was then applied to the hind paw to augment immobilisation of the digits further and to allow the animals to walk comfortably after recovery from anaesthesia.

**Harvesting of the tendons.** All the tendons were harvested seven days after operation using similar preoperative and anaesthetic procedures as described above. After incision of the skin, the tissue plane around the synovial sheath was dissected and the tendon/sheath complex was divided distal and proximal to the MCP joint and proximal interphalangeal joint, respectively. The stay suture was left in place to help with orientation of the tissue plane during histological processing. An assessment of the status of the wound and the appearance of the synovial sheath in each digit were made and recorded. Formaldehyde (10%) solution was used for the immediate fixation of the tissues.

**Electron microscopy.** A standard technique was used. Osmium tetroxide (1% solution) was used for the fixation of the tissue. Dehydration of tissues was with increasing concentrations of ethanol. The prepared specimens were embedded in Spurr’s resin (ERL 4206; Agar Scientific, UK).

**Immunohistochemistry.** Tissue specimens were treated with steam under pressure for two minutes to help to expose the antigens. The visualising system used was the biotin-streptavidin system (Biocare Medical, Concord, California, USA). The antigen control solution was applied to the sponge using a needle and syringe. By gently applying intermittent pressure, the solution of 5-FU was encouraged to spread throughout the synovial space via the sponge which was changed twice during the application time of five minutes. A mean volume of 0.4 ml of 5-FU was used per animal. The sponge was then removed and the area irrigated thoroughly with phosphate-buffered normal saline (PBS). The two control tendons were treated by irrigation with PBS.

**Statistical analysis of the combined data for the two observers comparing treated and control specimens using the Mann-Whitney rank-sum test.**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Observer 1 Control</th>
<th>Observer 1 Treated</th>
<th>Observer 2 Control</th>
<th>Observer 2 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>72.2 (3.7)</td>
<td>34.5 (2.4)</td>
<td>81.9 (2.6)</td>
<td>41.6 (2.0)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>102.2 (3.3)</td>
<td>61.1 (2.8)</td>
<td>117.2 (2.1)</td>
<td>70.8 (1.5)</td>
</tr>
<tr>
<td>RAM-11</td>
<td>102.3 (3.2)</td>
<td>89.5 (2.4)</td>
<td>110.4 (2.0)</td>
<td>104.8 (1.6)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>75.3 (4.5)</td>
<td>41.0 (2.6)</td>
<td>79.4 (1.9)</td>
<td>48.6 (1.9)</td>
</tr>
</tbody>
</table>

**Table II.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>RAM-11</td>
<td>&lt; 0.007</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
2) Vascular cell adhesion molecule-1 (VCAM-1; Santa Cruz Biotechnology Inc). This was a goat monoclonal and diluted 1:30.

3) Macrophages (RAM-11; Dako). This was a mouse monoclonal and diluted 1:25.

4) TGF-β1 (Serotec, Oxford, UK). This was a mouse monoclonal and diluted 1:100.

The secondary antibodies were biotinylated and were diluted in host animal serum to block non-specific staining as shown. They were:

1) for the Ki-67 staining, donkey-anti-sheep diluted 1:200;
2) for the VCAM-1 staining, donkey-anti-goat diluted 1:1500;
3) for the RAM-11 staining, horse-anti-mouse diluted 1:200; and
4) for TGF-β1 staining, horse-anti-mouse diluted 1:200.

After the primary incubation, the slides were washed in TBS for five minutes followed by secondary incubation and again washed in TBS for five minutes. Next they were incubated in the streptavidin/biotin complex for 35 minutes followed again with a wash for five minutes in TBS. The slides were then incubated in DAB solution for ten minutes. This was followed by staining in Mayer’s haematoxylin for four minutes, after which they were washed in running tap water. They were then rinsed in a 1% solution of acid/alcohol followed by washing in running tap water. The tissues were then dehydrated in a series of alcohols from 70% to 90%, 95% and then 100%. The slides were then treated in xylene and mounted for viewing.

Cell counting. Two independent and blinded observers examined the slides using a microscope and a 20 objective lens. Eight random fields per slide were chosen and those cells which stained positively, as judged by the obvious colouring, were counted in each field. Each observer chose ten representative slides for each marker (Table I).

Statistics. We used Sigma Stat software version 2.0 (Jandel Scientific, USA) to demonstrate that the means (n = 10) of the eight measurements per slide were normally distributed. All except the data for VCAM-1 counting were found to fit into a normal distribution. The VCAM-1 data were then subjected to non-parametric statistical analysis (Mann-Whitney U test). Comparisons between the data generated by the two observers showed that there was no significant (p > 0.1) difference between them. The means of the data for each observer were then pooled to allow overall comparisons to be made between the data for the treated and control tissue (n = 20). The combined data were not normally distributed for each marker and thus the statistical analysis was done using the Mann-Whitney rank-sum test (n = 20; Table II).

Results

The treated specimens were seen to be less oedematous and erythematous than the control specimens.

Figure 1 shows control and treated tissue from the synovial sheath taken from around the injured site. The gross cellular reaction of the untreated synovial sheath typifies its behaviour after injury in that there is a prolific cellular reaction. The cells present have the features of fibroblasts and macrophages and are metabolically active as judged by the presence of an abundance of rough endoplasmic reticulum and secretory granules. In comparison the response of the treated tissue is less cellular and the cells which are present appear to have less protein-synthesising machinery. Their features indicate, however, that they were still viable and there appears to be some synthesis of collagen in the treated tissue.

The typical staining patterns of treated and control tissue
are shown in Figure 2 which represents the staining patterns of the synovial sheath with the antibody Ki-67. This antibody recognises cells which are in all phases of the cell cycle apart from G0. Thus, cells which are metabolically active are stained. Direct comparisons can be made since the magnifications are the same. It can be seen that the treated tissue has a reduced number of active cells. This difference was shown to be significant (p < 0.001) using the Mann-Whitney rank-sum test. This may explain the reduced cell density in the treated tissue. There is an overall reduction in the expression of VCAM-1 in the treated group. Statistical analysis using the Mann-Whitney rank-sum test showed that the difference was significant (Table II; p < 0.001). This would indicate that the 5-FU was acting both as an antimetabolite and an anti-inflammatory agent. There was also a statistically significant (Table II, p > 0.007) difference between the density of macrophages in the treated and control tissue. Expression of TGF-β1 was also found to be significantly reduced (p < 0.001) in the treated tissue. This growth factor is expressed by endothelial cells as well as cells within the synovial tissue.

Discussion

In complex injuries of the limbs the restoration of function may be dependent on unpredictable processes relating to the healing of both the soft and hard tissues. Restoration of excursion of a tendon will ensure return of movement in the joint but unfortunately injured tendons heal with adhesions to surrounding structures which may prevent this. Loss of function of the tendon has significant repercussions when involving the long flexors of the hand particularly in zone II. Efforts to try to prevent the formation of adhesions have been attempted both experimentally and clinically. Recently, it has been shown that the synovial lining contains relatively aggressive fibroblasts, which may have a crucial role in the formation of these adhesions. Targeting of the synovial lining with 5-FU incorporated in the single-touch technique has been shown experimentally to be a feasible method to prevent adhesions from forming.

Although 5-FU is antiproliferative, simple reduction in the numbers of fibroblasts will not prevent the formation of adhesions; cell behaviour other than cell division has to be affected. The exact mechanism by which 5-FU produces its effect on cell behaviour is not entirely understood. The extracellular matrix is a storehouse of signals regulating the maturation, migration and division of cells. Also cells harbour mRNA in the cytoskeleton and either or all of these aspects could be affected by 5-FU. It is clear from our study that the single-touch technique in the reconstruction of tendons does not lead to overt cell death, but the metabolism and ‘set’ of the cells so treated are significantly affected. Since all the treated tendon units healed, the treated cells maintain some ultrastructural aspects which allow them to participate in wound healing.

The endothelium as well as the subintimal fibroblasts expresses VCAM-1, the level of which is increased during times of inflammation under the influence of certain cytokines. Assuming that the level of expression of VCAM-1 reflects the inflammatory response, it would appear that 5-FU reduces the level of expression of VCAM-1 and thus in this context 5-FU is acting as an anti-inflammatory agent. The inflammatory reaction to injury determines the recruitment of cells such as macrophages and fibroblasts, both of which are important in the initiation and perpetuation of the processes leading to adhesions. Thus, 5-FU appears to be acting directly and indirectly to reduce the noxious response of the synovial lining to injury. A single touch with 5-FU prevents the progression of cellular and molecular cascades which may lead to the formation of adhesions. The production of cellular proteins appears to be reduced by 5-FU as seen by the reduced mitochondria and rough endoplasmic
reticulum in the fibroblasts at the electron-microscopic level. Although there is a global reduction in the synthesis of protein this ensures both a reduction in the production of active proteins such as enzymes as well as limitation of the production of putative growth factors. Thus, the affected cell is limited in migration and has a reduced capacity to disorganise the extracellular matrix, both of which are requisites for fibrosis. It would seem that the cells are metabolically arrested. Reduced synthesis of protein alters the capacity of the cell to respond to putative growth factors since there will be fewer synthesised receptors for the growth factors to act through. In this context 5-FU is acting as an antimetabolite.

Our study has shown that treatment with 5-FU is not simply that of inhibiting cell proliferation but also of affecting important aspects of cell behaviour. This form of therapy may possibly be used clinically, but this can only occur if the healed tendons can be shown to take appropriate load after treatment. We are currently investigating this. A single-touch technique to reduce the formation of adhesions in the injured tendon using 5-FU appears to be a simple, focused, palatable, titratable and inexpensive means of refined treatment for the management of these complex cases.

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References