The Effect of 5-FU on the Expression of TGF – β1 in Cultured Tendon Cells

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Abstract

Objectives: This study investigated the effect of the treatment with 1 min exposures to 5-fluorouracil (5-FU) on the expression of Transforming Growth Factor – Beta 1 (TGF-β1) in cultured tendon cells.

Methods: Fibroblasts cultured from the flexor tendons of dog paws were treated with 3 different doses of 5-FU (control, 5-15-25 mgr/ml) for 1 minute. After 5-FU exposure the expression of TGF-β1 were tested by real time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) at 3rd and 7th days.

Results: There were no statistically significant differences in the expression levels of the TGF-β1 gene between the control group and all other groups on day 3 and 7 (p>0.05). However, when the percentage changes in the TGF-β1 gene expression on days 3–7 were compared, there were statistically significant differences and this was maximally observed with 89% +12 (p<0.05) in the group treated with 25-mg/ml 5-FU.

Conclusions: We conclude that 1 min. 5-FU application maybe sufficient to prevent adhesions in tendon healing by limiting the expression of TGF-β1.

Keywords: Adhesion, tendon cells, 5-fluorouracil, TGF-β1.

Introduction

Postoperative adhesion formation is still a challenging problem in flexor tendon healing, especially for tendons lacerated within the digital synovial sheath in the hand (1).

The experimental investigations of tendon healing have showed that tendons have the capacity to heal through an intrinsic and/or extrinsic tissue repair process (2-5). When the intrinsic healing process predominates, tendons heal with minimal adhesions. When the extrinsic healing process predominates thick adhesions form between the tendon and the surrounding tendon sheath, significantly limiting tendon excursion, leading to a poor clinical outcome.

Adhesion formation is an inflammatory response induced by the tendon flexor sheath in response to trauma and accentuated by total immobilization. If the balance between the brisk extrinsic healing and delayed intrinsic response could be changed it could reduce adhesion formation.

Various pharmacologic agents have been used to reduce adhesion formation in tendon healing. 5-FU is an antimetabolite used in cancer chemotherapy and appears to be an effective drug to prevent the adhesion formation in tendon healing. Blumenkranz et al found that 5-FU is able to inhibit the proliferation of the fibroblasts in cell cultures. Exposure of the surgical field to 5-FU produces a focal inhibition of scarring by inhibiting fibroblast activity (6,7,8). Moran et al. showed that a single 5 minute exposure of 5-FU applied topically at the time of flexor tendon repair reduced postoperative flexor tendon adhesions in a chicken model (9).

However, the effects of 5-FU on the tendon cells and growth factor expression are not clearly known. Growth factors are the chemical signals that direct the migration and proliferation of the tendon fibroblast during the healing process. TGF-beta (TGF-β) is a growth factor and a prominent regulator of tendon healing. TGF-β has been found to play a role in collagen production, angiogenesis, restoration of the gliding surface and even adhesion formation (10). Natsu-ume and Chang have found elevated levels of TGF-β known as profibrotic factor in injured tendons. These levels can remain elevated for up to eight weeks (11,12). Khan et al. showed increased expression of TGF-β1 by synovial sheath and epitenon cells (13). TGF-β1 is known to regulate the interactions between fibroblasts and ECM proteins. However, continuous synthesis of TGF-β1 causes uncontrollable fibrosis (14,15).

The aim of this project is to study the effect of 5 FU on the expression of TGF – β1 in cells derived from the canine flexor digitorum profundus tendon, in an
effort to better understand why 5-FU produces an improvement in tendon healing.

**Materials and Methods**

**Primary culture of dog flexor tendon cells**

The flexor digitorum profundus tendon from dog paws were harvested. The technique used to harvest the epitenon cells was modified from previously described procedures (16). The tendon was tied into a loop and suspended in a sterile solution of 0.25% trypsin in 20 mM HEPES buffer solution for 20 minutes at 37°C to harvest the epitenon cells. The supernatant was collected and centrifuged at 1132 rpm for 5 minutes to remove the trypsin solution, and the remaining epitenon cells were plated in 100 mm culture dishes. Half million epitenon cells harvested from the flexor profundus tendon of dog paws were treated with 3 different doses of 5 FU (control, 5-15-25 mgr/ml) for 1 minute. After 5 FU exposure the expression of TGF – beta 1 were tested by RT-PCR at 3rd and 7th days.

**RNA Isolation and Purification**

Total cellular RNA from control or 5 FU treated cells were extracted with TRIZOL reagent. Briefly, 5-10 x10⁶ cells were lysed in 1 ml TRIZOL reagent. After incubation of the homogenized samples for 5 minutes at 15-30 C, 0.2 ml of chloroform per 1ml of TRIZOL solution was added to the samples. Then the samples were centrifuged at 12000 rpm for 15 min. The colorless upper aqueous phase, where RNA remained of the mixture was transferred into a fresh tube. To precipitate the RNA, 0.5 ml of isopropyl alcohol per 1ml of TRIZOL reagent was added to the samples. After incubation at 15-30 C for 10 minutes, RNA was collected by centrifuging at 12,000xg for 10 minutes. Following centrifugation the supernatant was removed and 1ml of %75 ethanol were added to wash the RNA.

To purify DNA- free RNA, DNase and RNeasy column was used to exclude the contaminated DNA in the RNA. Extracted total RNA was treated with RNase free DNase in the solution of 50mM Tris-EDTA, 10mM MgCl₂, 0.1 mM EDTA, 7mM DDT and RNase inhibitor at 37 C for 1 hour. RNA was cleaned up with RNeasy kit. The amount of RNA was quantified with Ribogreen RNA Quantification Kit.

**Table 1.** The sequences Polimerase Chain Reaction Primers of Canine genes

<table>
<thead>
<tr>
<th>TGF-β1</th>
<th>LightCycler Probe Design Software</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>ACCATTCATGGCATGAACC</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAGATCCTTGCGGGAAGTC</td>
</tr>
</tbody>
</table>

**cDNA Synthesis**

Following cleaning up RNA, RNA was reverse-transcribed into single stranded cDNA with 1st Strand cDNA Synthesis Kit. Total RNA was reverse-transcribed into complementary DNA (cDNA) by incubation with of AMV reverse transcriptase in 20 microlitre of reaction buffer containing 3.2 microgram of random primers, 1ml MdNTPs, 5 mM MgCl₂ at 25C for 10 minutes then at 42 C for one hour. The amount of the dsDNA was quantified with PicoGreendsDNA Quantification Assay Kit.

**Real-Time Reverse Transcription PCR**

The expression of TGF – beta 1 was tested by real time RT-PCR. The PCR primers and probes were designed with LightCycler Probe Design Software 2.0. The cDNA sequences of genes were searched from NCBI. The PCR conditions were optimized with different concentration of magnesium and annealing temperatures. Real time PCR was performed on a LightCycler, a rapid thermal cycling instrument of Roche (Roche Diagnostics GmbH, Germany), in capillary glass tubes with the LightCycler-Fast Start DNA Master SYBR Green I kit (Roche).

**Statistical Analysis**

Statistical analyses were carried out using the SPSS (Statistical Package of Social Sciences) for Windows software program version 18.0. The Kruskal Wallis one-way analysisof variance was used, followed by the Mann- Whitney U test to evaluate the differences between groups. A p value of less than 0.05 was considered statistically significant.

**Results**

**Day 3 expression**

On day 3, there were no statistically significant differences in the expression levels of the TGF-β1 gene between the control group and all other groups after 1-min 5-FU treatments (p>0.05)(Fig. 1).
Figure 1: Day 3 expression

Day 7 expression

On Day 7, the expression levels of the TGF-β1 gene were lower than the levels of the controls in all groups; however, there were no statistically significant differences (p>0.05) (Fig. 2).

Figure 2: Day 7 expression

Days 3–7

When the percentage changes in the TGF-β1 gene expression on days 3–7 were compared, there were statistically significant differences between the control group and the other groups (5, 15, and 25 mg/ml) (p<0.05). This was maximally observed with 89% ±12 (p<0.05) in the group treated with 25-mg/ml 5-FU.In the groups treated with 5-mg/mL and 15-mg/ml 5-FU, the % 63 ± 14 and % 60 ± 4 percent reductions were determined respectively (Fig. 3).

Figure 3: TGF-β1 gene expression on days 3–7

Discussion

Adhesions due to scar formation between the tendon and surrounding tissues following flexor tendon injuries of the hand can lead to the loss of tendon gliding. Today, attempts to reduce postoperative adhesion formation have included low-friction surgical repair techniques, early postoperative rehabilitation, physical barriers to adhesion formation, tendon surface lubrication, and the use of pharmacological anti-adhesive reagents (17).

As better understanding of the complex molecular events of tendon healing evolves, it has been found that the best outcome requires effective pharmacological modulation in addition to attentive surgical techniques and rehabilitation. Various pharmacological agents have been investigated, but no agent has earned a clinical consensus as yet (18,19).

Although the exact mechanism of 5-FU remains unknown, its effects have inspired studies in which the agent was applied after tendon repair in attempts to limit adhesion formation (20).

Khan et al. investigated the effect of 50mg/ml 5-FU after a 5-min application in the partial laceration of rabbit tendons. They also examined the secretion of transforming growth factor beta 1 with immunohistochemical techniques. They showed that the expression of TGF-β1 was significantly reduced in the treated tissue at postoperative Day 7 (21).

However, Bulstrode et al. found that a 5-min 5-fluorouracil application had no significant effect on TGF-β1 secretion and TGF-β1 gene expression by
tendon fibroblasts (22). They used a 5-fluorouracil concentration of 25 mg/ml for gene expression experiments. Total RNA was isolated at 0, 6, and 24 h after exposure to 5-fluorouracil. They found an increase in gene expression within 24 h; however, after 24 h, the difference with the control group was not significant.

In our study, unlike that of Bulstrode et al., gene expressions were evaluated for three different doses: 5, 15, and 25 mg/ml of 5-FU with 1 min application, except in the control group. RNA isolation was also carried out at relatively late periods (days 3 and 7 after 5-FU application). We did not find a statistically significant differences in the expression of the TGF-β1 gene between any groups on days 3 and 7. These findings were consistent with the work of Bulstrode et al., as it appeared that 1-min 5-FU treatment with 5,15, and 25 mg/ml doses did not affect the expression of the TGF-β1 gene at a relatively late stage. However, during the waiting period up to 3 and 7 days, we observed that the cells of the control group proliferated more rapidly than the cells in the groups treated with 5-FU. Therefore, in the control group, the cell culture plates ultimately did not have enough space for cell proliferation, which might have been a limiting factor for the expression of the TGF-β1 gene. We think that if a lesser number of cells and/or larger culture dishes were used, the effects of 5-FU on TGF-β1 gene expression might be different.

On days 3–7, there were statistically significant decreases of TGF-β1 expression in all 5-FU treatment groups, which was maximally observed with 89% + 12 (p<0.05) in the group treated with 25-mg/ml. Based on these data, it can be assumed that a 25-mg/ml 5-FU dose could be effective in clinical practice. In addition, to the best of our knowledge, of all the in vitro and in vivo studies on tendon adhesion, 5-FU was applied for 5 min, whereas, in our study, the cells were treated for 1 min. A 5-min period can be quite a long wait time during surgery, and a 1-min, 25-mg/ml 5-FU dose might be sufficient to prevent adhesions clinically.

**Conclusion**

Consequently, the 5-FU seems to be a suitable agent to reduce adhesions. Further research to gain better understanding of its mechanism could assist in its transition into clinical practice.

**References**