Reduction in matrix metalloproteinase production by tendon and synovial fibroblasts after a single exposure to 5-fluorouracil


Phoenix Tissue Repair Unit, Department of Plastic and Reconstructive Surgery, University College London, London; and *Department of Plastic Surgery, The Queen Victoria Hospital, East Grinstead, UK

SUMMARY. This study investigated the effect of treatment with 5 min exposures to 5-fluorouracil (5-FU) on the production of matrix metalloproteinases (MMPs) by endotenon and synovial fibroblasts. Fibroblasts were grown from the flexor tendons of New Zealand White rabbits and were then exposed to varying concentrations (ranging from 0.25 mg ml\(^{-1}\) to 25 mg ml\(^{-1}\)) of 5-FU for 5 min. The treated fibroblasts were suspended in a three-dimensional collagen lattice. The conditioned media from these collagen lattices were then analysed for MMP production using gelatin zymography on days 1, 3 and 7 after treatment. In the majority of cases this treatment produced a dose- and time-dependent reduction in total MMP production by both cell lines, specifically in the production of MMPs 2 and 9. This reduction was significant for most concentrations (\(P<0.01\)–\(P<0.05\)) when compared to phosphate-buffered-saline-treated controls. We conclude that 5-FU may reduce adhesions by limiting the migratory capacity of synovial fibroblasts (extrinsic healing). © 2001 The British Association of Plastic Surgeons

Keywords: fibroblasts, tendon, adhesions, 5-fluorouracil, matrix metalloproteinases.

Materials and methods

Preparation of cells from explants

Cultures of tendon and synovial fibroblasts were grown from a total of 64 flexor digitorum profundus tendons harvested from eight New Zealand White rabbit hind paws. The synovial-sheath tissue was explanted as 5 mm\(^2\) sheets and placed in a culture dish under a glass cover slip to secure the tissue to the base of the dish. In a similar manner the core of the tendon (the endotenon) was cut into 5 mm\(^3\) pieces and explanted. After 1 week, fibroblasts had migrated out of the tissue onto the base of the culture dish and established colonies. These cells were
then passaged onto separate culture dishes. Cells were used between passages 3 and 6.

**Preparation of 5-FU concentrations**

The 5-FU (Allan Bull Laboratories, Warwickshire, UK) was divided into concentrations of 0.25, 0.5, 2.0, 10.0, 15.0 and 25.0 mg ml\(^{-1}\) with phosphate-buffered saline under sterile conditions. Phosphate-buffered saline alone was used as a control.

**Treatment of cultured fibroblasts**

Once the fibroblasts had achieved confluence on the culture dishes they were exposed to 5-FU treatment for 5 min at 37°C. Confluent cultures were exposed to the varying concentrations of 5-FU in duplicate batches. After treatment, the 5-FU was quenched with three phosphate-buffered saline washes over 15 min. The cells were then removed from the culture dishes and suspended in a collagen lattice as described below.

**Preparation of fibroblast-populated collagen lattices (FPCLs)**

The technique used has been described previously. Briefly, 4 ml of 2.28 mg ml\(^{-1}\) native acid-soluble rat tail type I collagen (First Link Ltd, UK; batch number 1797) was mixed with 0.5 ml of \(\times 10\) strength modified Eagles’ medium with Earl’s salts (GIBCORL) and neutralised by the drop-wise addition of 1 M NaOH until the colour of the solution turned permanently pink. Cell suspensions containing 500,000 cells in 0.5 ml of complete Dulbecco’s modified Eagles’ medium (DMEM) were prepared and added to the neutralised collagen gel. This solution was then poured into wells in a six-well plate and the collagen-gel–cell suspension was placed in a 37°C incubator and formed a lattice within 5 min. The FPCL was freed from the edge of the well and suspended in 5 ml of modified DMEM without phenol red (Gibco, UK) in order to eliminate its influence on protein measurement in the conditioned media. Conditioned media from the FPCLs was extracted on days 1, 3 and 7 with a pipette and dialysed overnight at room temperature using a specialised tubing (Sigma, Poole, UK; D-9527). This process is called exhaustive dialysis and is undertaken at 4°C against distilled water. The purpose of overnight dialysis is to de-salt the solution so that the resultant solution can be freeze-dried. The freeze-dried resultant was then stored at \(-20°C\). This process allows proteins to be retained so that they can be assayed.

**Gelatin zymography**

Analysis of gelatinases involved resolution by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, with gelatin as a substrate (10% tris-glycine buffered gel with 0.1% gelatin at pH 8.6) (Novex gels, R&D systems, Oxford, UK). The band pattern of the conditioned-media samples was analysed relative to purified MMP 2 (60, 62, 68 and 72 kDa; Sigma, Poole, UK) and MMP 9 (65, 84 and 92 kDa; Sigma, Poole, UK) standards, both at 10 µl (50 ng) per lane. In preliminary experiments this concentration was found to give the clearest bands on the zymogram, which were amenable to analysis by scanning densitometry. Conditioned media from the FPCL models from 1, 3 and 7 days was dialysed and stored freeze-dried. Reconstitution involved mixing 250 µl of protein with an equal volume of Laemmli sample buffer containing 0.5 M tris-HCl (pH 6.8), 10% (w/v) sodium dodecyl sulphate, 20% glycerol and 0.1% bromophenol blue. After standing for 15 min, 20 µl of the mixture was loaded onto the gel and electrophoresed for 1.5 h at constant voltage (125 V) and current (40 mA) in 0.24 M tris-base, 1.92 M glycine, 0.035 M sodium dodecyl sulphate until the tracker dye reached the gel base. After electrophoresis, gels were washed in 2.5% Triton X-100 for 30 min, to renature the proteins, and then in developing buffer containing 2 mM \(p\)-amino-phenyl-mercuric-acetate, to activate the pro-enzyme forms of the MMP species present in the gel. Coomassie blue R250 (Merck, Poole, UK) was used to stain the gels, and, after destaining with methanol, bands of enzyme activity could be identified as protein substrate lysis within the gelatin matrix, i.e. clear bands on a blue background indicating the presence of gelatinase. FPCL experiments were carried out in duplicate for both synovial- and endotenon-cell populations at days 1, 3 and 7 on four separate occasions.

**Quantitation by scanning densitometry**

Following de-staining, the zymogram gels were dried before scanning-densitometry analysis. Each gel was dehydrated in a solution containing 33% ethanol and 1% glycerol in distilled water for 20 min at room temperature. Then the gel was laid between two cellophane sheets in a plastic frame (Novex) and kept overnight in an upright position. Zymograms were scanned using Image master (Pharmacia Biotech, UK) to give the relative densities of bands compared to purified enzyme standards. The relative activities of the enzyme samples were expressed as optical densities. Before analysis of each gel, a stable baseline was set for each zymogram to ensure that the results were reproducible. These experiments were carried out in duplicate and the mean optical density values were obtained for each cell population at days 1, 3 and 7 after treatment. The experiments were carried out on four separate occasions.

**Statistical analysis**

The data were analysed using computer software (Sigmastat v. 2.0, Jandel Scientific, California, USA). This revealed that the results were not normally distributed and so the Mann–Whitney U-test was used to test whether there was a difference in optical densities between the cells that acted as controls and those treated with 5-FU.

**Results**

**Control 1: Phenol red-free DMEM with 0–10% foetal calf serum**

To control for any errors introduced into the analysis by the reagents used in the cell-culture studies in the zymograms,
phenol red-free medium without foetal calf serum was tested for MMP resolution using gelatin zymography. This did not show any MMP 2 or MMP 9 activity. With 10% foetal calf serum, MMP 2 and MMP 9 were present as faint bands (present in the serum) and did not affect the results of the assay for enzyme levels produced by the cells in the conditioned media.

Control 2: MMP 2 and MMP 9 production from synovial and endotenon cells without 5-FU treatment

Day 1. On day 1 the synovial fibroblasts produced significantly more MMP 2 than the endotenon fibroblasts ($P < 0.05$) (Fig. 1A). In contrast there was no significant difference in MMP 9 activity between the endotenon and the synovial fibroblasts ($P > 0.1$) (Fig. 1B).

Day 3. By day 3 the levels of production of both MMP 2 and MMP 9 had significantly increased for both endotenon fibroblasts and synovial fibroblasts compared with day 1 ($P < 0.01–0.5$). On day 3 synovial fibroblasts produced significantly more MMP 2 and MMP 9 than endotenon fibroblasts ($P < 0.01–0.05$) (Fig. 1).

Day 7. On day 7 synovial fibroblasts produced significantly greater amounts of MMP 2 ($P < 0.05$) and MMP 9 ($P < 0.01$) than endotenon fibroblasts. However, the increase in the rate of production between day 3 and day 7 was not as large as the increase between day 1 and day 3 (Fig. 1).

MMP 2 and MMP 9 activity of synovial fibroblasts after treatment with 5-FU

With increasing 5-FU concentration there was inhibition of MMP 2 and MMP 9 production by synovial fibroblasts (Fig. 2). No significant difference between the levels of treated and control MMPs was seen after treatment with 0.25–2.0 mg ml$^{-1}$ of 5-FU solution ($P > 0.1$). However, for concentrations in the range 10–25 mg ml$^{-1}$ there was a significant reduction in the amount of MMP 2 and MMP 9 production ($P < 0.05–0.01$).

Figure 1—The variation in (A) MMP 2 and (B) MMP 9 production by synovial (red bars) and endotenon (green bars) fibroblasts. There is a significant increase in the production of MMP 2 over time by both endotenon and synovial fibroblasts ($P < 0.05$). Importantly, the synovial fibroblasts produce significantly more MMP 2 and MMP 9 than the endotenon fibroblasts for days 1, 3 and 7 ($P < 0.05$). Note also that the magnitude of MMP 9 production was not as great as that of MMP 2 production. The MMP assays were carried out four times in duplicate and the histograms show the means of these data with standard-error bars.

Figure 2—The effects of a 5 min exposure to 5-fluorouracil (5-FU) on the production of (A) MMP 2 and (B) MMP 9 by synovial fibroblasts. No significant reduction in MMP 2 was observed for concentrations of 5-FU of less than 10 mg ml$^{-1}$ ($P > 0.01$). For 5-FU concentrations of 10 mg ml$^{-1}$ and above there was a significant reduction in MMP 2 production ($P < 0.05$). There was a dose-dependant reduction in MMP 9 production; statistical significance ($P < 0.05$) was seen only above 2 mg ml$^{-1}$. No MMP 9 was detected on day 1 after treatment with 5-FU at 25 mg ml$^{-1}$. The experiments were carried out four times in duplicate and the histograms show the means of these data with standard-error bars.
MMP 2 and MMP 9 activity of endotenon fibroblasts after treatment with 5-FU

The patterns of inhibition of MMP 2 and MMP 9 production were different for endotenon fibroblasts. MMP 2 production, like MMP-2 production by the synovial fibroblasts, did not show any significant reduction for 5-FU concentrations in the range 0.25–2.0 mg ml\(^{-1}\). For concentrations of 10 mg ml\(^{-1}\) and above there was a significant reduction in the production of MMP 2 (\(P < 0.05\)). The reduction in MMP 9 activity was not dose dependent; however, there was a significant reduction for all concentrations of 5-FU (\(P < 0.05\)). The experiments were carried out four times in duplicate and the histograms show the means of these data with standard-error bars.

Discussion

Fibroblast chemotaxis (and adherence to the substratum) is important in adhesion formation and is directly related to the fibronectin concentration. MMPs, particularly MMP 2 and MMP 9,\(^9\) are actively involved in extracellular-matrix degradation, which is a requisite for cell migration.\(^8\) Studies have demonstrated that synovial fibroblasts are a more reactive group of cells than endotenon fibroblasts.\(^18\) This study supports this hypothesis: synovial fibroblasts produced large quantities of MMPs compared to the endotenon fibroblasts. Previous studies have shown that MMP production is an integral part of fibroblast-mediated collagen contraction.\(^19\) MMPs are also involved in the release of growth factors and other important cytokines from the matrix, which itself acts like a storehouse.\(^20\) Thus, the function of these enzymes is not merely the simple destruction of the extracellular matrix, they may also be involved in the remodelling of the matrix through their specific but indirect influences on the cells via growth factors. A difference in the extent of MMP activity was noted, with MMP 2 being expressed to a greater degree than MMP 9. This suggests that the pathway for enzyme expression may differ between these two enzymes. It is not clear whether this difference occurs in the nucleus (gene expression) or in the cytosol (mRNA expression).

It is also not clear at which level in the orderly nature of protein synthesis 5-FU has the observed effect. The possible sites range from gene expression to the cytosolic modification of proteins. It may be that 5-FU causes a global reduction in cellular-protein synthesis and that this results in a non-specific reduction in MMPs. Regardless of the exact mode of anti-metabolic activity, it is clear that the ‘single touch’ technique results in a significant reduction in the production of these important enzymes. With judicious use of the ‘single touch’ technique it may be possible to address the imbalance between the intrinsic and extrinsic mechanisms of tendon healing.

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References


The Authors

Raj Ragoowansi MSc, FRCS, Specialist Registrar
St Andrew’s Centre for Plastic and Reconstructive Surgery, Broomfield Hospital, Court Road, Broomfield, Chelmsford, Essex CM1 7ET, UK.

Umraz Khan BSc, FRCS, FRCS(Plast), Specialist Registrar
Department of Plastic Surgery, The Queen Victoria Hospital, Holtye Road, East Grinstead, West Sussex RH19 3DZ, UK.

Robert Brown PhD, Lecturer
Duncan Angus McGrouther FRCS, MD, Head
Department of Plastic and Reconstructive Surgery, University College London, 1st Floor Charles Bell House, 67–73 Riding House Street, London W1N 8AA, UK.

Correspondence to Mr U. Khan, Specialist Registrar, Department of Plastic and Reconstructive Surgery, Chelsea and Westminster Hospital, Fulham Road, London SW10 9NH, UK.

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