The effect of 5-fluorouracil on Dupuytren fibroblast proliferation and differentiation*

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Summary

Introduction – Dupuytren's disease is a proliferative disease with contractile properties, prone to recur after surgery. Intra-operatively applied 5-fluorouracil has been used to avoid scar problems in the eye after glaucoma filtration surgery and was therefore investigated as a means to inhibit proliferation and myofibroblast differentiation in Dupuytren fibroblasts in vitro.

Method – Primary cell lines were obtained by explants from Dupuytren's tissue (n = 6), non-diseased palmar fascia from patients with Dupuytren's disease (n = 3) and carpal ligament from patients undergoing carpal tunnel release (n = 3). The effect of 5-fluorouracil on proliferation was assessed by cell counting. Myofibroblast differentiation, an integral part of Dupuytren's contracture, was investigated by staining for α smooth muscle actin, a marker for contractile cells, using immunohisto-chemical methods.

Results – A single exposure to 5-fluorouracil caused a sustained inhibition of proliferation in Dupuytren's and non-diseased fascia cultures, whilst the effect on carpal ligament cultures was transient. Untreated Dupuytren's fibroblasts exhibited the highest myofibroblast differentiation, whilst differentiation in non-diseased fascia cultures was shown to be proportional to cell density and virtually nonexistent in carpal ligament cultures. After 5-fluorouracil exposure, the differentiation was significantly reduced in Dupuytren's fibroblasts cultures, reduced at high cell densities in non-diseased fascia and unchanged in carpal ligament cell cultures.

Discussion – 5-fluorouracil inhibits both proliferation and myofibroblast differentiation in Dupuytren's cell cultures and may have a potential use as an adjuvant treatment to Dupuytren surgery in order to reduce the rate of recurrence and contracture.

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Dupuytren's fibroblasts / 5-fluorouracil

Résumé – Effet du 5-fluorouracile sur la prolifération et la différenciation des fibroblastes dans la maladie de Dupuytren.

Introduction – La maladie de Dupuytren est une pathologie proliférative aboutissant à la formation d'un contracture tissulaire, qui récidive souvent après chirurgie. L'application peropératoire de 5-fluorouracile a été utilisée pour éviter ce type de problème dans la chirurgie du glaucome. C'est pourquoi

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nous avons étudié in vitro l'effet du 5-fluorouracile sur la prolifération et la différenciation en myofibroblastes des fibroblastes de maladie de Dupuytren.

Matériel et méthodes – Des cultures primaires de fibroblastes ont été obtenues à partir d'une méthode utilisant l'explant de tissu de maladie de Dupuytren (n = 6), de fascia sain de patients ayant une maladie de Dupuytren (n = 3), ou de ligament de carpe chez de patients opérés pour un syndrome du canal carpien (n = 3). L'effet de 5-fluorouracile sur la prolifération des fibroblastes a été mesuré en comptant les cellules. La différenciation des fibroblastes en myofibroblastes, impliquée dans la pathogénie de la maladie de Dupuytren, a été évaluée par immuno-histochimie en effectuant un marquage avec anticorps spécifique de l’α-actine qui est un marqueur des cellules contractiles.

Résultats – Une exposition unique au 5-fluorouracile provoquait une inhibition prolongée de la prolifération cellulaire dans les cultures de maladie de Dupuytren et de fascia sain, alors que l'effet sur les cultures de ligament annulaire du carpe n'était que transitoire. Le degré de différenciation myofibroblastique le plus élevé était observé dans le fibroblastes de maladie de Dupuytren, alors que cette différenciation apparaissait proportionnelle à la densité cellulaire dans les cultures à partir de fascia sain, et quasi-inexistante dans les cultures à partir de ligament annulaire du carpe. Après ajout de 5-fluorouracile, cette différenciation diminuait significativement dans les fibroblastes de maladie de Dupuytren et dans les cultures à haute densité cellulaire à partir de fascia sain, alors qu'il n'y avait pas d'effet sur les cultures de ligament annulaire du carpe.

Discussion – Ces résultats suggèrent que le 5-fluorouracile inhibe à la fois la prolifération et la différenciation myofibroblastique dans les cultures de cellules de maladie de Dupuytren. Le 5-fluorouracile pourrait donc avoir un intérêt en tant que traitement adjuvant en complément de la chirurgie dans la maladie de Dupuytren, pour diminuer le taux de récidive et la contracture. © 2000 Éditions scientifiques et médicales Elsevier SAS

fibroblastes / 5-fluorouracile / maladie de Dupuytren

Dupuytren's contracture is a disease of fibroblast proliferation and can be divided into three histological stages according to Luck [1]. The initial stages of the disease are characterised by proliferation (Luck's stage 1) as is the woundbed after any operation, as part of the normal woundhealing. Hypothetically wound healing and Dupuytren's disease proliferation are therefore related processes and may be stimulated by pathogenetically common factors. It may therefore be speculated when operating on Dupuytren's disease, one initiates a recurrence by initiating the woundhealing process. The most common therapeutic problem in Dupuytren's disease is recurrence after surgery, defined as disease in an area previously operated for Dupuytren's disease. Published series report recurrence rate with the range of 34-71%, [2-13].

In 1968 it was observed that chemotherapy of cancer of the lymph nodes resulted in the disappearance of Dupuytren nodules and further clinical trials indeed confirmed the resolution of the hyperproliferative nodules, but that fibrotic cords persisted [14]. This observation suggested that Luck's stage I Dupuytren's disease was metabolically active and may be influenced by chemotherapy.

The use of 5-fluorouracil to inhibit fibroblast proliferation in the eye following surgery has been in clinical use for a long time. Initially 5-fluorouracil was used as postoperative injections into the eye; later as a peri-operative topical treatment [15-17]. Preclinical studies further support the use of chemotherapy to control fibroblast proliferation in woundhealing, as in vitro experiments illustrated that even short exposures to 5-fluorouracil have longterm effects on cell culture proliferation [18]. Animal experiments, showed a well localised effect in the eye affecting both proliferation and motility as measured by fibroblast outgrowth from biopsies taken after treatment [18, 19].

With the ultimate aim of reducing recurrence rates in Dupuytren's disease, it was therefore decided to investigate the effect of 5-fluorouracil on the proliferation of Dupuytren fibroblasts in vitro.

The other major problem in Dupuytren's disease is contracture, which is thought to be mediated by myofibroblasts. Fibroblasts acquiring the morphological
and biochemical features of smooth muscle cells, including the expression of α-smooth muscle actin, the actin isoform typical of vascular smooth muscle cells, are called myofibroblasts [20]. The distribution of myofibroblasts is widespread: in granulation tissue and in pathological tissue, such as liver cirrhosis, burn contractures, fibromatosis (including Dupuytren’s disease) and in cirrhotic cancers [21, 22]. Myofibroblasts have also been found in the apparently non-diseased fascia of Dupuytren patients together with high levels of collagen type III, indicating that the disease is widespread within the palmar aponeurosis [23]. Any fibroblasts can become a myofibroblast as it has been suggested that it is impossible to grow a myofibroblast free culture in vitro [24]. When fibroblasts metamorphose into myofibroblasts, they synthesise, like stimulated macrophages, very high amounts of plasminogen activators, which could be taken as a biochemical sign of evolution of contracture [25]. Myofibroblasts and vascular cells undergo apoptosis in the maturation from granulation tissue to scar and it has been proposed that it is only when granulation tissue cells are not eliminated pathological scarring develops [26]. This indicates that the further presence of myofibroblasts may perpetuate disease. Myofibroblasts are thus implicated as leading to contracture [20, 27] and it has been shown that the acquisition of smooth muscle like phenotypes correlates with increased contractility in Dupuytren fibroblasts [28]. Any effect diminishing myofibroblast differentiation could therefore be expected to be beneficial in Dupuytren’s disease.

**PATIENTS AND METHODS**

**Patients**

Primary cell cultures obtained from Dupuytren’s tissue, non-diseased fascia from the same hand and carpal ligament from patients undergoing carpal tunnel decompressions was used in the following experiments.

Dupuytren’s tissue was excised at operation for Dupuytren’s contracture and non-diseased fascia was taken as a biopsy from a macroscopically uninvolved area of the hand at the edge of the primary incision. Carpal ligament specimens were taken from patients without Dupuytren’s disease undergoing carpal tunnel release.

The patient data is summarised in Table I.

<table>
<thead>
<tr>
<th>Table I. Patient data.</th>
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<tr>
<td>Dupuytren tissue (n = 6, mean age = 65 years ± SD 8, all male, four primary, two recurrent)</td>
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<tr>
<td>Non-diseased fascia (n = 3, mean age = 65 years ± SD 16, all three male)</td>
</tr>
<tr>
<td>Carpal ligament (n = 3, mean age = 52 ± SD 9, two female and one male)</td>
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**Cell cultures and 5-fluorouracil**

The explant method was used in obtaining primary cell cultures from the harvested material, maintained at 37 °C, 5% CO₂ and covered with standard media (DMEM, Gibco). All cell lines used were below passage 4.

Cells were plated at a density of 40,000 cells/well in 6 well plates. Each experiment consisted of a single dose of 5-fluorouracil (David Bull Laboratories, Warwick) and was conducted in triplicate. At day 0 the cells were plated and allowed to settle for 24 hours, before being treated for 5 minutes with 5-fluorouracil at 2.5 mg/mL at day 1. Pilot experiments using serial dilutions of 0-10 mg/mL 5-fluorouracil showed that a concentration of 2.5 mg/mL did not increase the cell mortality, whilst still inhibiting proliferation, and was therefore chosen. After treatment cultures were washed with PBS (Phosphate Buffered Saline) before covered with media as normal. Cultures were enumerated at day 2, 7, 14, 21 and 30 after treatment. Cells were harvested and coloured with Trypan blue (Sigma Chemical Company, Poole, Dorset), which is pumped out of the live cell in normal conditions, thus any dead cell will appear blue in contrast to the living cells. The average of 3 grids on the haemocytometer was taken as the nearest accurate number of cells in the specimen.

The Wilcoxon signed rank test was used for statistical comparisons.
Monolayer cultures for immunohistochemical staining and 5-fluorouracil

Glass coverslips were sterilised by immersion in 70% alcohol, then placed in wells and allowed to dry under sterile conditions. Cells from Dupuytren’s tissue, non-diseased fascia and carpal ligament were seeded at a density of 40,000 cells per well in six-well plates, in triplicate for each experiment and allowed to settle for one day before treatment as above.

To fix the monolayer cultures, media was pippetted off and the cultures rinsed in PBS before suspension in ice cold Methanol for 10 minutes in the freezer (20°C). The coverslips were gently removed from the wells and fixed to histology slides with nail varnish before being stored in the freezer (20°C).

Staining for alpha smooth muscle actin

The primary antibody (anti alpha actin, Boehringer Mannheim Biochemica) was diluted as follows: 1.3 mL Phosphate Buffered Saline: 150 μL Bovine Serum Albumin (Life Technologies, Paisley, UK) solution: 50 μL anti alpha actin and kept on ice. Hundred μL of dilute antibody was added to each coverslip before they were incubated for 1 hour in moist conditions. Then the coverslips were first washed twice and then immersed in PBS for 10 minutes. The secondary antibody was diluted as follows: 3.59 mL PBS: 400 μL BSA: 400 μL propidium iodide: 10 μL FITC-conjugated secondary antibody (DAKO, Denmark) and kept on ice in dark. Hundred μL of diluted antibody was added and incubated in moist conditions at room temperature for 1 hour in the dark. The slides were washed and immersed in PBS for 10 minutes before mounting with DABCO. The slides were stored in the freezer (20°C).

Enumeration of alpha smooth muscle actin stained monolayer cultures

The cells were counted under an Ultra Violet light, rendering the propidium iodide nuclear stain bright orange and any alpha smooth muscle actin bright green.

Three times 300 cells were counted in each specimen and any α-smooth muscle actin positive cells were taken as a percentage of the total number of cells.

The Wilcoxon signed rank test was used for statistical comparisons.

RESULTS

Proliferation (figure 1)

Control cultures of Dupuytren, non-diseased fascia and carpal ligament fibroblasts showed no significant difference in proliferation in vitro under standard culture conditions. In 5-fluorouracil treated cultures the mitogenic response was proportionally inhibited to the concentration of 5-fluorouracil used. No increase in cell death was however seen following treatment with 2.5 mg/mL 5-fluorouracil for 5 minutes and this concentration was therefore used in the subsequent studies. No cultures exhibited a
significant difference in cell numbers between treated and un-treated experiments at day 2. The mitotic rate in Dupuytren and non-diseased fascia fibroblasts was significantly inhibited from day 7-30 ($p < 0.03$), but was only significantly reduced in carpal ligament cultures from day 7-21 only ($p < 0.02$). The proliferation of all treated cell lines was inhibited for the first two weeks following 5-fluorouracil treatment. After this carpal ligament fibroblasts recommenced proliferating, whilst both Dupuytren and non-diseased fascia fibroblasts remained inhibited, although to a lesser extent than previously. At day 30 the proliferation in carpal ligament fibroblasts was therefore not inhibited significantly.

**Differentiation (figure 2)**

Dupuytren fibroblast cultures maintained a relatively high proportion of myofibroblasts throughout the experiment compared with non-diseased fascia and carpal ligament cultures. In contrast cultures of non-diseased fascia fibroblasts, exhibited an ever increasing amount of myofibroblasts related to cell density and time and carpal ligament fibroblast differentiation remained low throughout the experiment.

5-fluorouracil decreased the of α-smooth muscle actin in Dupuytren fibroblasts to a significant degree throughout the duration of the experiment ($p < 0.003$), whilst differentiation in non-diseased fascia fibroblasts was significantly suppressed at day 30 only (day 2-21 $p > 0.05$, day 30 $p < 0.0039$). Carpal ligament fibroblast differentiation was not affected by 5-fluorouracil ($p > 0.8$).

**DISCUSSION**

Adjuvant treatment in conjunction with surgery for Dupuytren's disease may modify the Dupuytren fibroblast response to wound-healing and thus reduce the risk of recurrence and contraction.

Proliferative disorders in the eye prompted the search for a pharmacologic agent capable of selectively inhibiting the growth of rapidly proliferative cells without unacceptable toxic effects on normal cell populations [29]. The first experiments, like ours, investigated the effect of 5-fluorouracil and other neoplastic drugs on a cellular level. The therapeutic range of 5-fluorouracil was found to be broad and to have a potentially safer therapeutic index with a strongly dose-dependant inhibition of fibroblast proliferation than other neoplastic drugs investigated [29]. It is thought that 5-fluorouracil is converted enzymatically to the nucleotide 5-fluoro-2-deoxyuridine phosphate which is a potent inhibitor thymidylate synthetase. This effectively inhibits the conversion of uridylate to thymidylate. In the absence of adequate levels of phosphorylated deoxyribonucleotides of thymidine for incorporation in DNA, mitotic activity is reduced, and cellular proliferation ceases. This block, at the level of thymidylate synthetase, can be overcome by the addition of exogenous thymidine or folic acid [30]. However, the presence of these substrates does not notably alter the inhibitory effect of 5-fluorouracil, indicating that this agent probably has a mechanism other than just the inhibition of thymidylate synthesis [29].
The application of drug data derived from cell culture to clinical disease has many limitations [30]. Variables such as bioavailability, diffusional barriers, metabolic inactivation, excretion, drug resistance and enzyme induction prohibit simple extrapolation of cell culture data to in vivo experimental models of disease. Nevertheless, this basic approach to drug selection is invaluable.

Having identified the medium with which to inhibit proliferation, the remaining problem was which cell to target. The origin of the fibroblast responsible for Dupuytren’s disease has been heavily disputed. Some authors believe the Dupuytren fibroblast to be the same as those native to the (hand) palmar fascia and that the major phenomenon in Dupuytren’s disease is an increase in proliferating fibroblasts rather than an alteration in the type of fibroblasts [21]. Supporting this theory an increased ratio of type III to type I collagen within the palmar fascia even before clinical symptoms and signs appear have been suggested [32], but the same author have proposed that the origin of the cells within Dupuytren nodules may arise from the dermis rather than the pre-existing fascia [32]. Some authors [33] have speculated whether these cells are in fact myofibroblasts which lie in the dermis of specimens taken from dermofasciectomy patients, explaining the low recurrence rate after this operation [6], especially since the presence of myofibroblasts has been linked to recurrence [34, 35]. The presented experiments showed that myofibroblasts are also present in significant numbers in non-diseased fascia fibroblast cultures, especially at high cell densities.

Apart from being implicated in the contraction seen in Dupuytren’s disease [28], the myofibroblasts also has also been shown to produce collagen type III, I and V [36]. The myofibroblast appears to be a central cell in the pathology of Dupuytren’s disease and any inhibition of fibroblast differentiation toward myofibroblasts would thus be beneficial in the therapy of this disease. The benefit could ideally be derived not only in conjunction with actual contraction but also with regards to collagen production. In examining native cells to the hand, like carpal ligament and non-diseased fascia fibroblasts it becomes clear that the Dupuytren's disease exhibits significantly higher levels of myofibroblasts.

Post-operatively, the remaining non-diseased fascia is stimulated to proliferate, as part of the normal wound healing response and this leads to an increased proportion of myofibroblasts, resembling levels found in Dupuytren's disease. In vitro treatment with 5-fluorouracil not only inhibits the myofibroblast differentiation in Dupuytren fibroblasts but also the potential source of myofibroblasts in the non-diseased fascia.

A 5 minute topical application of 2.5 mg/mL 5-fluorouracil in culture was shown to inhibit cell proliferation for approximately 10-14 days after which the cells began to proliferate. This timing is extremely important since this is the exact time period for normal wound-healing. This mitotic response therefore provides cells for the formation of a scar. One could therefore hypothesise that if this initial proliferation is successfully inhibited in Dupuytren fibroblasts, the cellular basis for recurrence would not be formed. Comparing all 5-fluorouracil treated cell lines, proliferation in Dupuytren and non-diseased fascia fibroblasts was inhibited to a higher degree than carpal ligament fibroblasts, representing normal cells, which were seen to recover more rapidly. This indicates that normal cells are less affected by 5-fluorouracil treatment than abnormal. Therefore treatment with 5-fluorouracil targets the Dupuytren and the non-diseased fascia fibroblasts foremost.

\(\alpha\)-Smooth muscle actin production representing the metamorphosis of fibroblasts to myofibroblasts was shown to be significantly different in Dupuytren's disease, non-diseased fascia and carpal ligament. Dupuytren's disease fibroblasts expressed significantly more alpha smooth muscle actin than any of the other cell lines throughout the experiment. Non-diseased fascia was shown to increase the production of alpha smooth muscle actin with increasing cell density in culture, mimicking the initial hyperproliferative stages in Dupuytren's disease. This could potentially be seen as an indication of the capability of the non-diseased fascia to act as a source for recurrence in Dupuytren's disease. Unsurprisingly carpal ligament expressed very little \(\alpha\)-smooth muscle actin throughout the experiment. A short treatment with 5-fluorouracil was shown to inhibit the production of \(\alpha\)-smooth muscle actin to a significant degree throughout the experiment with
Dupuytren's disease fibroblasts, at day 30 in non-diseased fascia fibroblasts, but made no significant difference to the already small \( \alpha \)-smooth muscle actin production seen in carpal ligament fibroblasts. This decrease in myofibroblasts may be of use in the prevention of the contracture seen in Dupuytren's disease.

It is therefore suggested that by inhibiting both proliferation and myofibroblast differentiation in Dupuytren cell cultures 5-fluorouracil may have potential use as a possible adjuvant treatment to Dupuytren surgery in order to reduce the rate of recurrence and contracture.

**REFERENCES**


