THE EFFECT OF STEROIDS ON DUPUYTREN'S DISEASE: ROLE OF PROGRAMMED CELL DEATH

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This study compared the rates of proliferation and apoptosis of cells within nodules of Dupuytren's disease and nodules from patients that had been injected preoperatively with steroid (Depo-Medrone). It also compared the effects of steroids in apoptosis in cultured Dupuytren's cells and control fibroblasts from palmar fascia and fascia lata. Steroids reduced the rate of fibroblast proliferation and increased the rate of apoptosis of both fibroblasts and inflammatory cells in Dupuytren's tissue. Steroids also produced apoptosis of cultured Dupuytren's cells but not of palmar fascia and fascia lata cells.

INTRODUCTION

Dupuytren's disease is classified among the fibromatoses, and in its early stages is histopathologically similar to fibrosarcoma (Erdmann et al., 1995). It also has similarities to granulation tissue (Fitzgerald et al., 1999), and myofibroblasts are involved both in Dupuytren's disease and inflammatory conditions. In resolving inflammatory conditions, myofibroblasts disappear by means of programmed cell suicide (apoptosis), though in chronic inflammatory tissue, where excessive scarring develops, myofibroblasts persist (Desmouliere, 1995). This survival of the myofibroblasts is dependent upon factors controlled by inflammatory cells. Dupuytren's tissue also contains inflammatory cells such as monocytes and lymphocytes, which produce a variety of cytokines, arachadonic acid metabolites and reactive oxygen species (Baird, 1994).

Steroids have been used to treat Dupuytren's disease (Ketchum and Donahue, 2000), both to reduce nodule formation and progression to contracture and post-operatively to reduce oedema and facilitate rehabilitation (Naylor et al., 1994). However, steroids do not affect existing contractures (Naylor et al., 1994). Other studies have looked at the effect of 5-fluoruacil on Dupuytren's cells (Jemec et al., 2000) and suggested its potential as an adjuvant treatment to Dupuytren's surgery in order to reduce the rates of recurrence and contracture.

The regulation of inflammatory cell apoptosis is complex. This study looked at the effect of steroids on apoptosis in Dupuytren's tissue in relation to programmed cell death and proliferation rates of inflammatory cells and myofibroblasts. Control of this could reduce the rates of progression and recurrence.

METHODS

Immunohistochemistry experiments

With the approval from the local Ethical Committee, and after obtaining written consent, 32 patients with Dupuytren's disease were randomly allocated into two groups. Sixteen Dupuytren's patients (14 men and two women) were not treated preoperatively, while 16 Dupuytren's patients (12 men and four women) were preoperatively injected with steroid. The average age was 54 (range 42 72) years and 57 (35–75) years in the untreated group in the steroid-treated group.

The group treated with steroid had 0.5 ml (20 mg) Depo-Medrone (methylprednisilone acetate, Upjohn) injected into the perinodular area of the palm 5 days before surgery. All patients were at a comparable stage of Dupuytren's contracture activity, as assessed by clinical and subsequent histological examination. Surgery was performed routinely under regional anaesthesia and tourniquet control. Four other patients undergoing routine carpal tunnel decompression had a representative sample of palmar fascia excised for comparison to the Dupuytren's tissue. Surgery was performed under identical conditions, again with regional anaesthesia and tourniquet control. The samples of excised Dupuytren's tissue and palmar fascia control tissue were placed immediately in 10% neutral buffered formalin solution.

All procedures were performed at room temperature unless otherwise stated. The tissue samples were dehydrated sequentially through a gradation of alcohol and histoclear solutions before embedding in paraffin wax. The tissue blocks were then cut and 7-μm sections were placed on 3-Aminopropyl triethoxy-silane (APES) coated slides and air-dried. Sections were then immunostained by the double sandwich technique employing the avidin–biotin alkaline phosphatase complex. Briefly, sections were de-waxed with histoclear then rehydrated through graded alcohol solutions to 0.05 M Tris Buffer system pH 7.2 (TBS), then pre-treated with trypsin (Sigma) for 15 minutes at 37°C. After three washes with TBS pH 7.2, the sections were incubated with the primary antibody at room temperature for 1 hour. The sections were then incubated with biotinylated secondary antibody (1:60 Sigma Immuno Chemical solution), for 45 minutes at room temperature and washed three times with TBS pH 7.2. Finally, the sections were incubated with extravidin alkaline phosphatase (1:400 solution Sigma Immuno Chemical), for 45 minutes at room temperature and were washed three times with

TBS pH 7.2. Bound alkaline phosphatase was visualized using BCIP/NBP (5-Bromo-4-Chloro-Indolyl Phosphate/Nitro Blue Tetrazolium) at room temperature until visualization was complete or background changes were becoming evident. This gave a bluish/black/brown colour depending on the intensity of reaction. The section was placed in distilled water to stop this reaction. The sections were then counterstained with neutral red (0.5%) and mounted in 50% glycerol solution with a coverslip. Control negative sections were run without primary antibody. The antibodies used were Lewis Y (1:60 dilution Sigma) and Ki 67 (1:60 dilution, DAKO) antigen markers for apoptosis and proliferation respectively. Cells which stained positively for Lewis Y and Ki67 were counted on six random × 140 magnification fields of comparable areas from three consecutive sections from every sample.

Ki67 is a nuclear protein which is expressed in proliferating cells (Rose et al., 1994). Lewis Y is a carbohydrate marker associated with the process of apoptosis, but not with cell proliferation or necrosis (Hiraishi et al., 1993). Statistical analysis between counts was performed by the Mann–Whitney U-test.

Flow cytometry experiments

Written consent was obtained and specimens were collected from six patients (five men and one woman) undergoing palmar fasciectomy for Dupuytren's disease. Their average was 62 (range 49 80) years. Control palmar fascia tissue was obtained from two patients (one man and one woman), aged 63 and 52 years, undergoing carpal tunnel decompression. All these operations were done under regional anaesthesia with tourniquet control of the limb. In addition, samples of normal fascia lata tissue were obtained from four patients undergoing routine hip arthroplasty surgery (two men and two women, average age 80 years, range 72–94).

Each sample was diced into 1-mm fragments and digested at 37°C in collagenase (0.1%) and DNAse (0.01%) for 3 hours, followed by trypsin (0.05%)/ethylene diaminete tetracetic acid (EDTA) (0.02%) for 1 hour. Filtration yielded a cell suspension in which residue enzymatic activity was neutralized by washing in medium containing 10% foetal calf serum (Gibco). Adherent cells were grown up to sub-confluence. In the Dupuytren's cell cultures, the adherent cells were predominantly fibroblasts.

Half of the cells from the cultures from Dupuytren's tissue, palmar fascia and fascia lata had soluble steroid (10^{-3} mmol) added to the culture medium 14 hours prior to harvesting. They were then harvested and suspended in binding buffer at a concentration of 1×10^6 cells/mL. The steroid used in the cell culture experiments was Solu-Medrone (Upjohn) as it was miscible with the medium. The concentration of 10^{-3} M was chosen as

this induced observable changes characterized by intense cell-surface activity followed by cell shrinkage, with subsequent condensation of chromatin in a crescent-shaped manner along the nuclear envelope. These changes are consistent with apoptosis. The level of apoptosis was measured by Annexin V (R & D systems) binding and exclusion of propidium iodide was ascertained with flow cytometry.

Statistical analysis of data, where appropriate, was performed by the Mann–Whitney U-Test.

RESULTS

Immunohistochemistry

In sections of control Dupuytren's tissue, the cells staining positively for the Ki67 proliferation marker were predominately fibroblasts and were located at the periphery of many of the active nodules. In particular, the density of positively staining cells was increased in the vascular perinodular fibro-fatty layers. There were a few positively stained cells within the centre of nodules and very few within the cords leading away from the nodules. The Lewis Y marker for apoptosis stained in a similar pattern to Ki67 for fibroblasts in the control Dupuytren's tissue, but in marginally reduced frequency. A few macrophages and occasional lymphocytes also stained positively for Lewis Y.

In the steroid-treated Dupuytren's tissue the frequency of Ki67-expressing cells was generally decreased, and in particular the peripheral tissue normally associated with proliferation of fibroblasts exhibited the greatest reduction. Although there was an increase in the number of Lewis Y-expressing fibroblasts at the periphery of the steroid-treated Dupuytren's tissue (Fig 1), the cells demonstrating the most marked increase in Lewis Y-expression were macrophages. There was also a slight increase in the relative numbers of lymphocytes staining positively for Lewis Y. The changes in cell marker frequency are summarized (Table 1).

Flow cytometry

Steroids produced different effects on Annexin V binding in the three different groups of cells analysed by flow cytometry. In the first group of six separate Dupuytren's cell cultures, addition of the steroid significantly increased ($P < 0.01$) the percentage of cells purely binding Annexin V from 1.7% to 18%. In the palmar fascia cells group, two culture groups were used. Following the addition of steroid, Annexin V binding altered from 4.3% to 3.9%. In the tensor fascia lata cell group, the steroid added to the cultures altered average Annexin V binding from 4.3% to 5.3%. Thus, only the Dupuytren's cells had a statistically significant increase in Annexin V binding on the addition of steroid (Table 2).
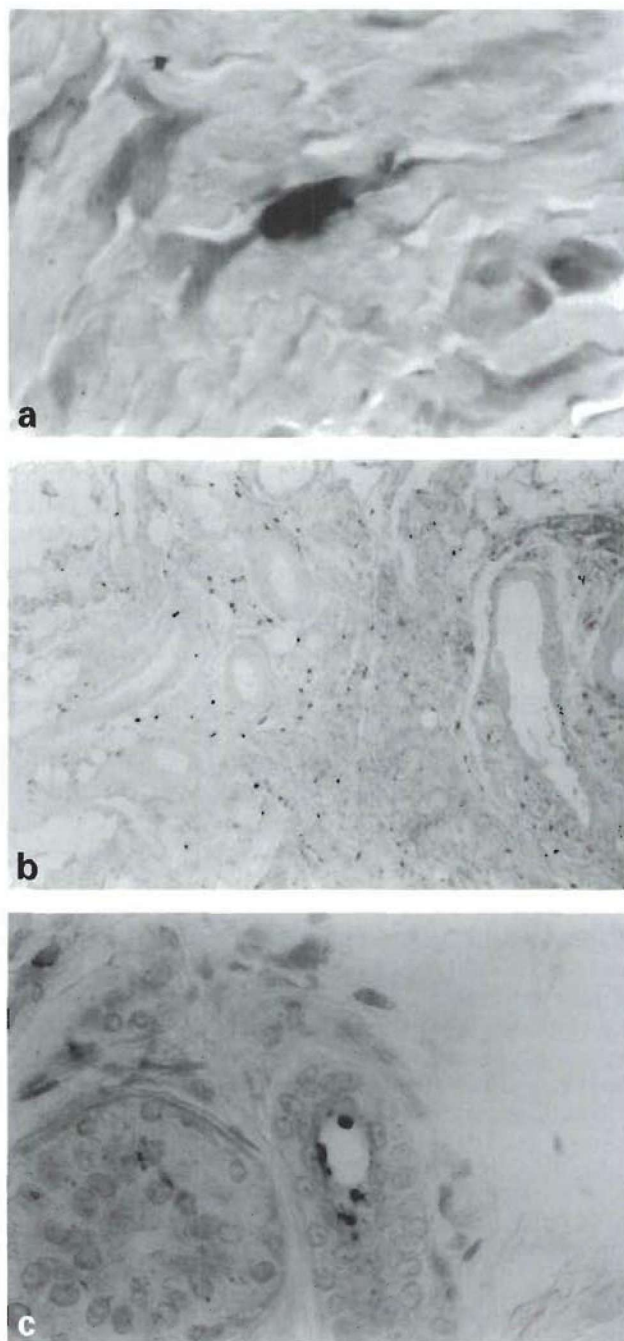
Fig 1   Illustrative sections of Dupuytren's tissue showing changes produced by preoperative treatment with steroid (stained by immuno-histochemistry for Lewis Y antibody). (a) Myofibroblast undergoing apoptosis (× 480). (b) Macrophages and fibroblasts undergoing apoptosis (× 120). (c) Inflammatory cell apoptosis (× 480) (neutral red counter-stain).

Table 1—Average cell counts per field of cells staining for Lewis Y and Ki67 antigens in sections of Dupuytren's tissue

Antibody	Control Dupuytren's tissue	Steroid-treated tissue
Lewis Y	1.1 ± 1.5	1.9 ± 2.1*
Ki67	1.3 ± 2.1	0.8 ± 1.7*

*$P < 0.05$.

Table 2—Apoptosis rates in cell cultures of Dupuytren's tissue, palmar fascia tissue and fascia lata tissue

	Untreated (%)	Treated (%)
Dupuytren's cells ($n = 6$)	1.7 ± 0.9	18 ± 6.9*
Palmar fascia cells ($n = 2$)	4.3	3.9
Tensor fascia cells ($n = 2$)	4.3	5.3

*$P < 0.001$.

a cure and recurrence is common, with a rate of up to 68% in some series (Leclercq and Tubiana, 1993).

Much work has been undertaken to understand why Dupuytren's disease recurs. Recent work has focused on inflammatory cells (Andrew et al., 1991; Baird et al., 1993) and there may be an imbalance between the control of cell proliferation and programmed cell death (Jemec et al., 1999). Dupuytren's disease has been compared to wound healing (Fitzgerald et al., 1999), the control of which is orchestrated by inflammatory cells that produce a variety of growth factors, which are also found in Dupuytren's disease (Baird et al., 1993). In the resolution phase of wound healing, cytokine secretion decreases and the number of inflammatory cells decreases, particularly monocytes. This is achieved without the release of lysosomal enzymes and without further tissue damage or inflammation. The mechanisms involved in this are not fully determined but histological evidence suggests self-programmed cell death (apoptosis) (Savill et al., 1989). We suggest Dupuytren's contracture represents a reduced rate of inflammatory cell apoptosis within the tissue, leading to sustained myofibroblast proliferation.

Many factors are involved in the control of inflammatory cell apoptosis. These include the composition of the extracellular matrix, growth factors, inflammatory cytokines, interleukins and nitric oxide. Inflammatory cells adhere to substances such as collagen, fibronectin and laminin and this prevents their apoptosis (Mangan et al., 1993). Abundant levels of fibronectin are present in Dupuytren's tissue (Meek et al., 1999; Tomasek et al., 1999), which acts as a natural ligand for inflammatory cell survival. Growth factors including TGF-β1 are extremely potent at recruiting inflammatory cells and promoting their survival. The pro-inflammatory cytokines, IL-1 and tumour necrosis factor alpha (TNFα) are also independently able to inhibit monocyte apoptosis (Maier et al., 1996; Mangan et al., 1993). The increased expression of all these cytokines by

DISCUSSION

The treatment for Dupuytren's contracture is surgical and has a significant morbidity due to skin necrosis, neurovascular damage and joint stiffness. Surgery is not

Dupuytren's tissue cells has previously been demonstrated (Baird et al., 1993) and thus Dupuytren's tissue has great potential for reducing the rate of inflammatory cell apoptosis.

Steroids have been used to treat the nodules of Dupuytren's disease (Ketchum and Donahue, 2000) and may act by multiple pathways. Previous work has demonstrated reductions in fibronectin, pro-inflammatory cytokine production and TGF-β1 expression in Dupuytren's tissue after treatment with steroid (Meek et al., 1999), all of which may play a role in inducing inflammatory cell apoptosis.

Increased free radicals formation has been implied in Dupuytren's contracture (Murrell, 1992). Recent evidence implicates nitric oxide as a primary inducer and a second messenger in intracellular signal transduction pathways involved in inflammatory cells' apoptosis (Shimaoka et al., 1995; Um et al., 1996). The effect of steroids on inducible nitric oxide synthetase (iNOS) and nitric oxide (NO) formation in Dupuytren's disease is unknown. Licensed drugs now exist which alter free radical formation and may offer another avenue of medical intervention to prevent progression or postoperative recurrence of Dupuytren's disease, without affecting wound healing.

In summary, our study has demonstrated steroid-induced apoptosis in Dupuytren's tissue. The relevance to Dupuytren's disease is that this may be a mechanism to prevent contracture progression or postoperative recurrence. Any adjuvant therapy to prevent recurrence would be of great aid, particularly to patients that are young, have ectopic lesions and a strong family history. Other drugs can also influence inflammatory cell apoptosis and these may prove highly effective in the treatment of fibrous proliferative conditions.

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