Effect of diacetyl rhein on the development of experimental osteoarthritis. A biochemical investigation

BY S. L. CARNEY

Osteoarthritis Department, Lilly Research Centre Ltd., Lilly Research Laboratories, Erl Wood Manor, Windlesham, Surrey, GU20 6PH, U.K.

Summary

Objective: To investigate the effect of diacetyl rhein (DAR) on the synthesis, turnover and composition of cartilage in an experimental model of osteoarthritis in beagle bitches.

Design: Osteoarthritis was induced in mature beagle bitches by the transection of the cranial cruciate ligament. Six animals received DAR 20 mg/kg daily for 11 weeks. A matched group received empty capsules daily for the same period. At 11 weeks, articular cartilage was examined for the ratio of the 6:4-sulfated disaccharides of chondroitin and the tissue concentration of hydroxyproline and glycosaminoglycan. In addition, labeling studies were performed to estimate the effect of DAR on proteoglycan synthesis and turnover.

Results: DAR had no effect on body weight or food consumption but induced a mild diarrhea and slightly increased the incidence of vomiting. DAR tended to reduce proteoglycan synthesis, however, DAR did reduce proteoglycan turnover in the femoral cartilage. DAR produced changes in the composition of the osteoarthritic cartilage that could only partly be accounted for by changes in hydration and/or swelling. In addition, it was noted that induction of osteoarthritis increased the ratio of chondroitin 6-sulfated to chondroitin 4-sulfated disaccharides; DAR reduced the ratio in tibial plateau cartilage from osteoarthritic joints compared with untreated tissue from osteoarthritic joints. DAR showed moderate reduction on the biosynthesis of proteoglycans. DAR also produced a reduction in proteoglycan turnover from all anatomical areas compared with non-treated controls in both the lateral and medial femoral condyles.

Conclusions: DAR was well tolerated by the experimental animals, but did not produce significant changes in the synthesis or turnover of proteoglycans. The slight reduction in proteoglycan synthesis may prove to be biologically significant after chronic dosing. DAR's effects on the hydroxyproline and glycosaminoglycan content suggest, however, that it must influence the swelling of cartilage and loss of glycosaminoglycan. This indicates that small changes can translate to significant differences in cartilage composition over an 11-week time period.

Keywords: Diacetylrhein, Osteoarthritis.

Introduction

OSTEOARTHRITIS is a disease that affects many joints causing changes to all of the joint structures particularly the cartilage, bone and capsule. The disease is commonly not well-managed pharmaceutically [1], and those compounds that are commonly used are analgesics or nonsteroidal anti-inflammatory agents (NSAIDs). Both approaches are palliative and are particularly unsuccessful in treating more severe disease. Of more interest recently, are agents that have disease-modifying characteristics (DMARDs—disease-modifying anti-rheumatic drugs [1]) or that are chondroprotective [2, 3], in that they in some way prevent cartilage destruction or prevent cell death. Although there have been many claims for disease-modifying activity of various agents, few have been subjected to rigorous experimental investigation or indeed clinical trial to assess disease modification.

One agent, diacetyl rhein [DAR; Figure 1(a)] has attracted particular attention of late [4]. The molecule is an anthraquinone which on administration is completely deacetylated to the structure shown in Figure 1(b), which is known as rhein. Rhein has a diverse pharmacology, and is capable of inhibiting glucose uptake [5, 6], protein synthesis [7], protease activity [8] and various other biochemical processes. As a result, it has been suggested that DAR may be useful as an anti-cancer agent. What is perhaps more intriguing is the ability of DAR to improve the symptoms of osteoarthritis, by (it is claimed) disease modification. It has been shown that DAR does not work in the same way as orthodox anti-inflammatory agents [4, 9, 10], yet can be shown in humans to produce relief by about 2-3 weeks after the commencement of treatment [4, 11, 12].
DAR significantly inhibited the histological development of lesions in animal models of osteoarthritis [13]. This has been demonstrated in spontaneous disease in the guinea-pig where no surgical or chemical agent has been used to initiate the disease process [14, 15]. It is not clear, however, how DAR exerts its anti-osteoarthritic effects. This study was intended to examine the effects of DAR on the early morphological and biochemical changes [16, 17] produced by the induction of osteoarthritis in a well-established surgical model. One of the approaches adopted in this study was to examine the effect of DAR on the production of mimotopes recognized by the monoclonal antibody 7-D-4. These mimotopes have been shown to be greatly elevated in osteoarthritic tissue [18, 19]. We have supposed that such mimotopes may be related in some way to a repair mechanism that generally is ineffective. We proposed that successful drug intervention may have some effect on the expression of these glycosaminoglycan mimotopes. Eventually we might hope that the level of these mimotopes could be used to quantitate beneficial change due to drug treatment. It was hoped that a diverse approach such as this might allow investigation of the potential molecular events which DAR may modulate, and hence, give clues as to how future anti-osteoarthritic drugs should be designed.

**Materials and methods**

**EXPERIMENTAL ANIMALS**

Twelve beagle bitches, weighing between 10.5 and 13.6 kg were divided into two groups, one group received DAR (mean weight 12.2 kg, range 10.9–13.6 kg) and the other received empty capsules as controls (mean weight 12.2 kg, range 10.5–13.3 kg). The dogs were maintained for a ‘predose’ period of 4 weeks before surgery and dosing commenced to establish that the dogs had become acclimatized and that any abnormalities (blood chemistry, etc.) could be identified. This was necessary because the animals were brought in from a remote site and may not have settled in the new environment. During this period the animals received no medication. Following the predose period, all dogs were subjected to division of the cranial cruciate ligament by a blind incision [20–22]. The anterior cruciate ligament of the right leg was divided and the contralateral leg provided the control ‘normal’ tissue. Two dogs (970 and 966) in the DAR group bled significantly and required suturing. In the control group, only dog 978 showed any significant bleeding, and required a single suture. The dogs were housed singly in pens with low dividers, which reduced the frequency of the dogs rising on their back legs to see their kennel mates or handlers entering the room. Experience has shown that this reduces the severity of the arthritic lesions. Because DAR seems to be most effective in animal models in reducing the rate of progression of mild or moderate disease, it was intended to have as mild a lesion as possible to maximize the effect of the molecule.

The animals in the DAR group received DAR at a dose of 20 mg/kg daily in capsule form, the first dose being administered the day after surgery. The control group received only empty capsules. The dogs were observed over the next 11 weeks for various indications (body weight, food consumption) and clinical signs (liquid faeces, colored urine, colored faeces, vomiting). Hematology and blood biochemistry were examined at weeks -2, 5 and 10 from the commencement of dosing. All dogs were killed 11 weeks after surgery by a single lethal dose of sodium thiopentone. On receiving the joints, they were washed, doused with ethanol then each knee flexed and opened under aseptic conditions. Excess soft tissue and muscle was removed before photography of the distal femurs. Full-thickness blocks (i.e. down to bone) were removed using scalpels and fixed in buffered formalin for light microscopy. Tissue removed for

![](image.png)

**FIG. 1.** (a) The structure of diacetyl rhein. (b). The structure of rhein, the totally deacetylated metabolite of diacetyl rhein.
light microscopic examination were from the same sites used for the tissue culture experiments.

PREPARATION, LABELING AND MAINTENANCE OF CARTILAGE CULTURES

The cartilage was removed and prepared for explant culture essentially as outlined by Carney et al. [22] from the following anatomical sites: the medial tibial plateaux, both operated and control (MTP A, MTP N); the lateral tibial plateaux, both operated and control (LTP A, LTP N); the medial femoral condyles, both operated and control (MFC A, MFC N); the lateral femoral condyles, both operated and control (LFC N, LFC A); and the patellar grooves (intercondylar fossa), both operated and control (PG A, PG N). The tissue was distributed into preweighed vials containing Dulbecco's modified Eagle's medium (DMEM) (containing 10% (v:v) fetal calf serum (FCS); 1 ml). After distribution, the slices were pulse labeled with a mixture of two radiotracers each at 10 ~Ci/ml. The two isotopes used were 35SO42-, as inorganic Na235SO4 and [3H]-proline. In this study we have examined only the results from the sulfate incorporation experiments. The results of protein labeling studies will be given elsewhere. Pulse labeling was for 4h. Following pulse labeling, the tissue was washed (2x1 ml) with sterile PBS, then resuspended in DMEM containing 10% FCS for a chase period of 48 h. All pulse and chase protocols were performed in an humidified incubator at 37°C gassed with CO2/air (5/95%). The radioisotope was incorporated almost exclusively into sulfated glycosaminoglycans, and hence, will enable us to determine the fate of newly-synthesized proteoglycans.

After pulse and chase protocols had been completed, the chase medium was removed and stored at -20°C. The tissue slices were extracted for 48 h with the following extractant: 4 M GuCl/50 mM acetate pH 5.8 containing the following protease inhibitors: 100 mM aminocaproic acid; 10 mM EDTA; 5 mM benzamidine.HCl. When extraction was completed the guanidine was removed and stored at -20°C. The tissue slices were washed with water (2-3x1 ml) and then digested with pepsin at 50 mg/ml in 0.5 M acetic acid (1 ml) for 48 h at 4°C. The pepsin digest was then removed and stored as for all the previous samples and the tissue slices finally digested with papain (13 mg/100 ml in 0.5 M acetate buffer pH 6.5 containing 20 mM cysteine HCl and 1 mM EDTA; 1 ml) for 16 h at 65°C. Chase samples and guanidine extracts were dialyzed exhaustively against deionized water. All samples were then counted in a liquid scintillation spectrometer. The complete removal of unincorporated isotope by dialysis was confirmed by chromatography on Sephadex G-25 of several random samples in each dialysis batch. The total counts in the chase medium, guanidine extract, pepsin digest and papain digest were determined. The total incorporation (pmoles SO4/mg cartilage/h) could then be determined as could the rate of release of newly synthesized proteoglycan and/or protein into the medium over a 48-h period. This figure was determined by calculation of the percentage of the total incorporated counts found in the chase medium.

IMMUNOSTAINING OF CARTILAGE SECTIONS USING 7-D-4

Blocks of cartilage were removed from all of the areas of the joint and were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. The sample blocks were then embedded in lowicryl K4M resin at -35°C. Sections (0.5 μm) were cut and collected on gelatin coated slides. The sections were stained using 7-D-4 at a dilution of 1 in 50 in 1% rabbit serum and were visualized using the Amersham DAB enhancement kit (cat. no. RPN 1174).

Following staining, the preparations were photographed, taking care to use the same area in each preparation. The full-depth photographs were taken and a grid devised so that one could examine the amount of staining in four separate equal-depth zones from the articular surface to the subchondral bone. A scoring system was devised so that the degree of staining, where 0 indicated no staining and 4 virtually complete staining, in any area could be determined. A panel of 12 observers then scored the photographs individually in a double-blinded manner and the results were then decoded, collated and examined.

ANALYTICAL METHODS

Uronic acid was determined by the method of Bitter and Muir [23], which had been scaled down for microtiter plate analysis [24]. Hydroxyproline was estimated by the method of Stegemann and Stalder [25], again scaled down for microtiter plate analysis. Radioactivity was determined by liquid scintillation spectroscopy, using a scintillant to sample ratio of >4.

Analysis of the sulfate isomers of chondroitin was by capillary electrophoresis essentially as outlined by Carney and Osborne [26]. Electrophoresis was performed on guanidine extracts that had been subjected to chondroitinase digestion under
the following conditions. Samples were adjusted to 0.01 M tris acetate pH 7.3 by the addition of a concentrated stock buffer. Chondroitinase ABC (30 mU) was added and the samples incubated for 1 h. The samples were then electrophoresed at 15 kV, 40°C in 40 mM phosphate/40 mM sodium dodecyl sulfate (SDS)/10 mM sodium tetraborate pH 9.0 using Applied Biosystems 270A and 270HT instruments. The capillary was monitored at 232 nm and the areas under each isomer peak estimated by a SpectraPhysics SP4290 integrator.

Throughout this study, statistical significance was assessed by a one-tailed Walsh test for paired data. This test was particularly appropriate for the type of paired data generated in this investigation, which represents a large number of single observations and not multiple comparisons which would have benefitted from analysis of variance.

**Results**

**GROSS ANATOMY**

At the time of death, all animals had large medial and lateral femoral osteophytes, as described previously. There were no cartilage lesions obvious on any of the joint surfaces. This result is consistent with many other experiments performed in our laboratory [14, 18, 19, 22]. It appeared that in this study, the development of osteophytes preceded the development of cartilage lesions. There were no apparent differences in the gross pathology of DAR-treated arthritic joints compared with control arthritic joints. The contralateral control joints of all animals showed no signs of any pathological change.

**BLOOD BIOCHEMISTRY**

The following parameters were examined: glucose, protein (albumin and globulins), urea, creatinine, alkaline phosphatase, alanine transaminase, aspartate transaminase, bilirubin, Na⁺, K⁺, Ca²⁺, P, Cl⁻ and cholesterol. All values fell within normal ranges for the whole duration of the study. There was no difference between the DAR treated group and the control group at any time during the study. In addition, a full hematology screen was performed, again there were no significant differences between the DAR treated and control animals, and all values fell within the normal range.

**CLINICAL SIGNS**

There was no significant effect of the DAR treatment upon body-weight. As might be expected, treatment with DAR also had no significant effect on food consumption over the test period. Colored urine and feces were observed in most animals for the duration of the study but was not clinically significant because the pink coloration was due to rhein (the major metabolite of DAR) which at slightly basic pH is intensely purple. At more acidic pH, rhein is yellow in color. The only detrimental side effects encountered in the DAR group was mild diarrhea and an increased incidence of vomiting. All dogs experienced at least one episode of diarrhea, the minimum observed was one episode during the study and the maximum was 10 episodes per animal. The mean value was four episodes per animal during the test period. Only one animal in the control group, and three in the treated, had episodes of vomiting. The bulk of the observations (13/21) were due to a single animal, dog 982. The diarrhea was a known side effect of DAR administration in humans [2] and was not unexpected, reports of vomiting were not, however, reported from human trials and may be of little consequence because the bulk of observations were from a single animal.

**PROTEOGLYCAN SYNTHESIS**

Table I shows the results of the incorporation of ³⁵SO₄ into proteoglycans from control and osteoarthritic cartilage from joints obtained from DAR-dosed and non-dosed animals. It can be seen that DAR produces a significant reduction (P < 0.047) in the rate of synthesis of nonoperated control tissue from the medial tibial plateau only. It can be noted, however, that in all cases, the synthesis rates in the DAR-treated animals were all lower than non-DAR treated, although not significantly so. DAR also produced a significant reduction in the rate of synthesis of arthritic tissue from the lateral femoral condyle compared with arthritic tissue from non-treated animals. It was perhaps of interest that only two anatomical sites in animals not dosed with DAR showed statistically increased rates of synthesis in osteoarthritic compared with controls. These were the medial tibial plateau (P < 0.031) and the lateral femoral condyle (P < 0.031).

**PROTEOGLYCAN TURNOVER**

Table II shows the results of the effects of DAR on the turnover of proteoglycans (assessed as the release of labeled proteoglycans or their fragments into the maintenance medium during the chase period as a percentage of total radiolabeled proteoglycans). DAR had a significant effect on the
Table I
Effect of diacetyl rhein (DAR) on the rate of incorporation of sulfate into proteoglycans from cartilage from control and experimental osteoarthritic joints

<table>
<thead>
<tr>
<th>Site</th>
<th>Control</th>
<th>Arthritic</th>
<th>Control</th>
<th>Arthritic</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTP</td>
<td>0.90 ± 0.27</td>
<td>1.21 ± 0.36†</td>
<td>0.73 ± 0.17*</td>
<td>0.78 ± 0.18</td>
</tr>
<tr>
<td>LTP</td>
<td>0.83 ± 0.29</td>
<td>0.81 ± 0.24</td>
<td>0.68 ± 0.16</td>
<td>0.60 ± 0.23</td>
</tr>
<tr>
<td>MFC</td>
<td>1.01 ± 0.78</td>
<td>1.16 ± 0.48</td>
<td>0.62 ± 0.21</td>
<td>1.10 ± 0.27‡</td>
</tr>
<tr>
<td>LFC</td>
<td>0.72 ± 0.19</td>
<td>1.02 ± 0.34†</td>
<td>0.59 ± 0.26</td>
<td>0.71 ± 0.32*</td>
</tr>
<tr>
<td>PG</td>
<td>0.74 ± 0.26</td>
<td>1.22 ± 0.92</td>
<td>0.56 ± 0.28</td>
<td>0.63 ± 0.20</td>
</tr>
</tbody>
</table>

The effect of DAR on the synthesis of proteoglycans from osteoarthritic and contralateral control joint cartilage. MTP refers to the medial tibial plateau; LTP refers to the lateral tibial plateau; MFC to the medial femoral condyle; LFC to the lateral femoral condyle and PG to the patella groove. The figures in the columns refer to the incorporation of sulfate in pmols SO₄/h/μg hydroxyproline. The values show the mean ± 1 S.D.

The figures marked * in the DAR-treated column indicate a statistically significant (P < 0.047) reduction in incorporation compared with the corresponding non-DAR treated column. Figures marked † indicate a statistically significant (P < 0.031) increase in arthritic values compared with corresponding controls. Figures marked ‡ indicate a significant (P < 0.016) increase compared with corresponding DAR-treated controls. Significance was determined in all cases using a one-tailed Walsh test for paired data.

reduction of turnover in the femoral condylar compartments. In this study, significant increases in the turnover from arthritic compared with control was seen in only the lateral tibial plateau and medial femoral condyle which probably reflects a less-severe development of the disease process in these animals. We would suggest that this may demonstrate our original premise that altered housing conditions may reduce the severity of the disease. As was seen in the previous section on proteoglycan synthesis, although DAR only produced significant reductions in turnover in osteoarthritic tissue from the femoral compartment, DAR reduced the values from all areas compared with the corresponding non-dosed tissue, whether arthritic or control.

HYDROXYPROLINE CONCENTRATION

Table III shows that there was a highly significant decrease in the hydroxyproline concentration of arthritic tissue compared with controls. Hydroxyproline contents were determined after culture. Swelling of the osteoarthritic tissue could account for the significant reduction of hydroxyproline in osteoarthritic tissue compared with contralateral control tissue. This is consistent with other observations which have demonstrated an increase in hydration and an increased ability of osteoarthritic cartilage to swell. DAR is capable of preventing this increase in both the medial and lateral tibial plateaux and the patellar groove. In these areas, there was a statistically significant increase in the concentration of hydroxyproline in the DAR-treated osteoarthritic tissue compared with non-dosed arthritic tissue. There was no statistical difference between DAR-treated control and osteoarthritic concentrations of hydroxyproline at these sites, although there was a significant difference in the femoral condyles.

GLYCOSAMINOGLYCAN CONCENTRATION

There were significant decreases in the concentration of glycosaminoglycan (GAG) per wet weight of tissue from osteoarthritic compared with control cartilage in non-DAR treated joints (Table IV), paralleling the findings for hydroxyproline shown in Table III. However, there was no significant difference in the values for non-DAR treated arthritic tissue compared with DAR-treated arthritic tissue. This contrasted with the data for hydroxyproline, where both tibial
Table II

Effect of diacetyl rhein (DAR) on the turnover of newly-synthesized proteoglycans from cartilage obtained from control and experimental osteoarthritic joints

<table>
<thead>
<tr>
<th>Site</th>
<th>Non-DAR treated Proteoglycan turnover (% of total synthesized present in medium after 48 h)</th>
<th>DAR treated Proteoglycan turnover (% of total synthesized present in medium after 48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTP</td>
<td>21.38 ± 5.44 22.33 ± 4.83</td>
<td>16.35 ± 7.49 17.47 ± 6.09</td>
</tr>
<tr>
<td>LTP</td>
<td>19.08 ± 5.53 28.60 ± 12.6†</td>
<td>17.40 ± 10.2 17.00 ± 8.38</td>
</tr>
<tr>
<td>MFC</td>
<td>15.32 ± 8.16 20.02 ± 6.59‡</td>
<td>13.58 ± 6.29 13.17 ± 4.18**</td>
</tr>
<tr>
<td>LFC</td>
<td>18.82 ± 9.63 20.63 ± 7.41</td>
<td>13.75 ± 7.42 16.30 ± 11.00*</td>
</tr>
<tr>
<td>PG</td>
<td>16.27 ± 6.84 16.37 ± 7.69</td>
<td>13.37 ± 5.85 13.65 ± 6.85</td>
</tr>
</tbody>
</table>

The effect of DAR on proteoglycan turnover. Abbreviations are exactly as in Table I. The figures show the mean ± 1 S.D. of the percentage of radio labeled proteoglycan in the chase medium as a proportion of total incorporation. Values marked * indicate a significant (P < 0.047) decrease in the DAR-treated osteoarthritic group compared with corresponding non-DAR-treated osteoarthritic tissue and values marked ** indicate a similar change of higher significance (P < 0.031). The figures in the non-DAR treated arthritic column marked † and ‡ indicate a significant increase compared with non-DAR treated controls (P < 0.031 and 0.047, respectively). Significance was determined in all cases using a one-tailed Walsh test for paired data.

Table III

Effect of diacetyl rhein (DAR) on the concentration of hydroxyproline from cartilage of control and experimental osteoarthritic joints

<table>
<thead>
<tr>
<th>Site</th>
<th>Non-DAR treated Concentration of hydroxyproline (µg/mg wet weight cartilage)</th>
<th>DAR treated Concentration of hydroxyproline (µg/mg wet weight cartilage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTP</td>
<td>12.82 ± 1.29*** 9.79 ± 1.59</td>
<td>13.63 ± 14.25 14.25 ± 2.40††</td>
</tr>
<tr>
<td>LTP</td>
<td>17.85 ± 6.04** 11.71 ± 1.24</td>
<td>17.94 ± 3.57 15.13 ± 4.85†</td>
</tr>
<tr>
<td>MFC</td>
<td>18.08 ± 1.95*** 12.68 ± 1.46</td>
<td>19.89 ± 7.28*** 14.66 ± 4.62</td>
</tr>
<tr>
<td>LFC</td>
<td>22.79 ± 8.41*** 12.41 ± 1.66</td>
<td>22.73 ± 14.51*** 17.79 ± 10.44</td>
</tr>
<tr>
<td>PG</td>
<td>19.97 ± 2.07*** 14.21 ± 2.66</td>
<td>22.00 ± 10.14 19.96 ± 8.94††</td>
</tr>
</tbody>
</table>

The effect of DAR on the concentration of hydroxyproline as µg/mg wet weight of cartilage. Abbreviations are exactly as in Table I. The values represent mean ± 1 S.D. Figures marked ***, ** indicate a statistically significant (P < 0.016, P < 0.031 respectively) increase in the values for control tissue compared with corresponding arthritic values. Figures marked ††, † indicate a statistically significant (P < 0.031, P < 0.047 respectively) increase in the DAR treated arthritic tissue compared with corresponding non-treated arthritic tissue. Significance was determined in all cases using a one-tailed Walsh test for paired data.
Table IV

Effect of diacetyl rhein (DAR) on the concentration of glycosaminoglycan from cartilage of control and experimental ostearthritic joints

<table>
<thead>
<tr>
<th>Site</th>
<th>Non-DAR treated</th>
<th>DAR treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of GAG (µg/mg wet weight cartilage)</td>
<td>Concentration of GAG (µg/mg wet weight cartilage)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Arthritic</td>
</tr>
<tr>
<td>MTP</td>
<td>75.47 ± 18.07</td>
<td>62.73 ± 17.12***</td>
</tr>
<tr>
<td>LTP</td>
<td>100.79 ± 31.71</td>
<td>67.14 ± 27.25***</td>
</tr>
<tr>
<td>MFC</td>
<td>91.94 ± 26.40</td>
<td>62.83 ± 9.21***</td>
</tr>
<tr>
<td>LFC</td>
<td>93.40 ± 58.17</td>
<td>60.29 ± 15.94**</td>
</tr>
<tr>
<td>PG</td>
<td>90.13 ± 9.77</td>
<td>69.96 ± 19.95**</td>
</tr>
</tbody>
</table>

The effect of DAR on the concentration of glycosaminoglycan as µg/mg wet weight of cartilage. Abbreviations are exactly as in Table I. Values represent mean ± 1 S.D. Figures marked ***, ** indicate a statistically significant (P < 0.016, P < 0.031 respectively) decrease in the values for arthritic tissue compared with corresponding control values. Figures marked † indicate a statistically significant (P < 0.047) increase in DAR treated arthritic tissue compared with DAR treated controls. Significance was determined in all cases using a one-tailed Walsh test for paired data.

not be solely due to a prevention in swelling and/or hydration.

CHONDROITIN 4- AND 6-SULFATION

Table V shows that, as a result of induction of osteoarthritis, there is an increase in the proportion of the 6-sulfated isomer of chondroitin compared with the 4-sulfate. This increase is statistically significant in all but one (medial femoral condyle) of the areas examined. DAR produced decreases in the proportion of 6-sulfate in arthritic tissue of all areas, however this was significant only in the lateral femoral condyle. DAR also produced reductions in control tissue in all areas examined, however this was significant only in the lateral tibial plateau and lateral femoral condyle. It is interesting also that with the exception of the medial tibial plateau, there was no significant difference in the DAR-treated arthritic compared with the non-DAR-treated control, whereas as previously mentioned with one exception these were all significant in the untreated group.

Table V

Effect of diacetyl rhein (DAR) on the proportion of the 6-sulfated isomer of chondroitin sulfate as a percentage of the total sulfated disaccharides

<table>
<thead>
<tr>
<th>Site</th>
<th>Non-DAR treated</th>
<th>DAR treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chondroitin 6-sulfate (% of total chondroitin)</td>
<td>Chondroitin 6-sulfate (% of total chondroitin)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Arthritic</td>
</tr>
<tr>
<td>MTP</td>
<td>66.10 ± 0.83</td>
<td>66.10 ± 2.19*</td>
</tr>
<tr>
<td>LTP</td>
<td>64.40 ± 2.61</td>
<td>66.40 ± 3.00**</td>
</tr>
<tr>
<td>MFC</td>
<td>64.15 ± 2.22</td>
<td>67.72 ± 1.82</td>
</tr>
<tr>
<td>LFC</td>
<td>64.14 ± 2.86</td>
<td>65.31 ± 2.73*</td>
</tr>
<tr>
<td>PG</td>
<td>61.03 ± 2.82</td>
<td>64.44 ± 3.69**</td>
</tr>
</tbody>
</table>

The effect of DAR on the proportion of the 6-sulfate isomer of chondroitin sulfate. Figures refer to the percent of total chondroitin. Values show mean ± s.d. Figures marked ***, * indicate a statistically significant (P < 0.031, P < 0.047, respectively) increase in non-treated arthritic tissue compared with non-treated control. Values marked †, indicate a statistically significant (P < 0.016) reduction in DAR treated arthritic tissue compared with non-treated arthritic tissue. Values marked §, §§ indicate a statistically significant (P < 0.016, P < 0.031, respectively) reduction in the DAR treated control compared with non-treated control tissue. Values marked ‡, indicate a statistically significant (P < 0.047) increase in DAR treated arthritic tissue compared with DAR treated control tissue. Significance was determined in all cases using a one-tailed Walsh test for paired data.
THE EFFECT OF DAR ON THE EXPRESSION OF 7-D-4 EPITOPE IN NORMAL AND ARTHRITIC TISSUE

Fig. 2(a) shows the effect of induction of osteoarthritis on the expression of the 7-D-4 epitope in tissue from the medial tibial plateau. The results were established by histological scoring by a method outlined in the text in the Materials and Methods section. (■), Normal; (□), arthritic. (b) The effect of treatment with diacetyl rhein (20 mg/kg/day; 9 week study period) on the expression of 7-D-4 epitope in osteoarthritic tissue from the medial tibial plateau. The results were obtained by histological examination of tissue sections as in Fig. 2(a). (■), Arthritic; (□), arthritic (DAR treated). (c) The effect of diacetyl rhein (20 mg/kg/day; 9 week study period) on the expression of 7-D-4 epitope in normal (contralateral control) tissue from the medial tibial plateau. The results were obtained by histological examination of tissue sections as in Fig. 2(a). (■), Normal; (□), normal (DAR treated).

Discussion

DAR has an interesting history in that it was initially developed as a treatment for kidney stones by Charles Friedman, because when deacetylated to rhein, the molecule is capable of chelating metal ions, in particular calcium [27]. In this respect rhein shares structural similarities with other compounds that can bind calcium, many of which are useful in the study of cartilage and bone, for example, alizarin and tetracycline. Quite serendipitously, one of Friedman's patients obtained great relief from his arthritis as a result of treatment, and hence, the molecule was investigated with some success for the treatment of osteoarthritis. It was claimed that DAR behaved as an anti-inflammatory analgesic. There have been various publications supporting this view [28, 29] but quite clearly if rhein is behaving as an anti-inflammatory agent it is showing an atypical profile because it is slower in action than a standard NSAID (e.g. tenoxicam) [4] indicating that perhaps its effect on
pain may be due to the drug having a more fundamental, disease modifying effect. Moreover, it has been shown that DAR increases the synthesis of prostaglandins [29], which is in contrast with most NSAIDS although it must be stated that this would not be definitive evidence that the molecule was not acting as an anti-inflammatory agent. In support of the theory that DAR has a mode of action that is not related to its analgesic or anti-inflammatory properties, is that a very closely related entity, doxycycline, has been shown in animal models of osteoarthritis [30] to be effective in preventing disease progression. This may indicate that both act on a related mechanism because they are chemically related, and doxycycline has not been reported to have any analgesic or anti-inflammatory action.

It was thought that DAR may have a novel mode of action that may be useful in the treatment of osteoarthritis. This study was intended to examine the effect of DAR upon the synthesis and turnover of proteoglycans in order that we might understand the mode of action of this drug in more detail in order to develop more effective, better tolerated anti-arthritic agents.

In this study we noted a drug-induced diarrhea which could be attributed to the stimulation of prostaglandin synthesis [29], producing increased peristalsis because this effect can be inhibited in isolated guinea-pig ileum by treatment with indomethacin. Vomiting was another adverse reaction, however, this was infrequent and not noted in clinical trials in humans [4]. None of the other parameters examined (body weight, food consumption, blood biochemistry) showed any change from normal. A review of the literature, however, showed a more disturbing side effect that is possessed by rhein and other related anthraquinones. In various in vitro test systems, hydroxyanthraquinones have been shown to be tumour-promoting agents, in particular rhein has been demonstrated to exhibit weak clastogenic activity [30-34]. These properties seem to be related to the presence of free hydroxyl groups particularly in the 1, 8 position as in rhein [33]. It was for this reason that we abandoned our interest in DAR as an anti-arthritic agent because the potential for tumour promotion in an elderly, chronically-dosed population was too great. However, it should be possible to prevent the clastogenic effects in rhein by substituting the free hydroxyl groups with metabolically stable groups.

DAR has been shown to be beneficial in the treatment of osteoarthritis in humans, but its mode of action is unclear. Although DAR has a rich and varied pharmacology, it was not clear whether any or all of the described properties of this compound were responsible for its disease modifying characteristics. Because synthesis and turnover are energy requiring processes it is entirely possible that restricting glucose to the chondrocyte may reduce both. In addition, the restriction of glucose may reduce the build up of lactic acid around the chondrocyte. Because cartilage is avascular, the increased levels of lactate produced may prove toxic to the cell because it has no mechanism for clearance per se. Certainly DAR, and other anthraquinones of the DAR series, are capable of inhibiting glucose uptake (S.L. Carney, in preparation) and this property could be related to the ability of these compounds to inhibit turnover in an in vivo model of turnover in guinea-pigs [35]. Although in this study, statistical significance was not always reached, small differences may be biologically significant when one considers the length of duration of the arthritic disease in humans. It may also be that DAR may have more effect (see Tables I and II) on highly stimulated cells, and hence, would not achieve significance in this study since the degree of enhancement of synthesis and turnover were more moderate than one would normally aim to achieve with this model.

Our intention in this study was to develop a mild osteoarthritis in these animals because we believed that this would maximize our chances of demonstrating an effect due to DAR. In retrospect, we should perhaps have tried to develop the most severe arthritis we could achieve because in this experiment we were unable to obtain the degree of difference in osteoarthritic cartilage compared with controls that we would normally expect. This made interpretation of the differences due to drug treatment much more difficult. It has been my experience with this model that housing can significantly affect the natural history of the pathology. Animals that spend a lot of time on their hind legs (e.g. jumping up to see handlers and their kennel mates) develop a more severe disease than those animals that do not spend a lot of time on their hind legs. This was the reason for choosing cages with low dividers in this study.

Although one may be able to offer explanations for the behaviour of DAR at the molecular level in osteoarthritis, it is still difficult to rationalize this with the observed clinical benefit observed in human trials. Improvement based upon pain relief and functional indices indicated that DAR required greater than 6 weeks treatment [1]. Such a
change however, may suggest that as has been proposed, DAR works not on the inflammatory component of the disease, but in fact produces benefit by a fundamental change in cartilage and bone metabolism. Certain findings of this and other studies would suggest that this was the case. Most conclusively, there was a reversal in the osteoarthritis-induced change in the ratio of the sulfated disaccharide isomers of chondroitin. Such changes in the ratio of chondroitin sulfate isomers has previously been described in spontaneously developing osteoarthritis in guinea-pigs [35].

It was not possible to histologically demonstrate that DAR had an effect on the expression of 7-D-4 epitopes. It has been previously shown that as a result of induction of osteoarthritis there was a significant increase in the abundance of these epitopes [18, 19]. This has been confirmed by this study. The observation that there was no reduction in the epitope levels in DAR-treated animals and there may be a suggestion that DAR may even increase the levels, lends some support to the idea that 7-D-4 expression may reflect an attempted repair process. It may be that DAR could enhance this natural process, and hence, have its clinical effect. To prove this it would be interesting to examine the effects of DAR in models of repair produced by intra-articular papain injection [37].

This study has shown that although DAR can produce changes in cartilage metabolism and composition in experimentally-induced osteoarthritis in the dog, such changes are small in magnitude and reflect the exception rather than the rule. However, some of the changes are of interest and could point to potential long-term effects of the molecule. Intuitively, one might assume that the effects on turnover and hydroxyproline concentration may be in some way related to the beneficial effect. It is possible, however, that some other aspect of DAR pharmacology may be responsible for clinical improvement.

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