Protective effect of androgens against inflammation induced cartilage degradation in male rodents

J A P Da Silva, J-P Larbre, T D Spector, L A Perry, D L Scott, D A Willoughby

Abstract
Objectives—Rheumatoid arthritis (RA) is a disease which predominantly affects women. Interestingly, low serum androgen levels and clinical improvement with androgen replacement have been reported in male patients. The aetiopathogenic role of sex hormones in arthritis and their potential long term effects on joint destruction and disability remains unclear, however. This study was designed to investigate the potential influence of sex hormones on inflammation induced cartilage degradation in male rodents.

Methods—An in vivo model of cotton wrapped cartilage implants was used to assess the effects of androgen, oestradiol, and progesterone on inflammation induced cartilage degradation, and in vitro techniques were used to investigate the direct actions on cartilage metabolism and cytokine production in male animals.

Results—Orchidectomy resulted in accelerated cartilage damage which was reversed by replacement of physiological levels of androgens. Granulomatous tissue from castrated male rodents produced higher amounts of interleukin 1. Sex hormones reduced spontaneous proteoglycan loss in vitro but did not interfere with the effects of interleukin 1 on cultured cartilage.

Conclusions—Androgens appear to protect cartilage from inflammation induced breakdown in male animals. These results support a pathogenic role for hypo-androgenism in rheumatoid arthritis and suggest that long term androgen replacement may help prevent joint damage and disability.

Methods
IN VIVO CARTILAGE DEGRADATION
Castration and sham operation
Mature male BALB/c mice (Tuck & Sons, United Kingdom) aged 9 to 10 weeks were used, age matched in each experiment, and kept under controlled light conditions (14 hours light, 10 hours dark). All surgical procedures were performed under intraperitoneal anaesthesia with 0.07 ml of combined Hypnorm (Janssen) and Hypnovel (Roche) in water (1:1:2, v/v). Orchidectomy was carried out via a single abdominal incision after dissection and ligation of the spermatic cord. Sham operated animals had a similar operation but the gonads were replaced in the abdomen after exposure. Animals were left to recover for two weeks before further use.

Preparation of cartilage
Male Wistar rats 150–170 g (Tuck & Sons) were killed and both femoral head cartilages (FHCs) were dissected free of bone and pooled in Hank’s balanced salt solution (HBSS) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. The cartilages were thoroughly washed in HBSS, trimmed out of any adherent tissue in a laminar flow cabinet and left overnight in Dulbecco’s modified Eagle’s medium (DMEM), without phenol red supplemented with antibiotics as above, at
37°C in a humidified incubator (5% CO2/95% air) before further use for implantation or culture.

**Cartilage implants**

Femoral head cartilages were aseptically prepared for implantation by wrapping in sterile surgical gauze (Softsorb, Vernon-Carus Ltd, United Kingdom) (5±0.15 mg weight) moisturised with HBSS. Each mouse received a bilateral subcutaneous implant of either wrapped or non-wrapped FHC each side of the dorsal hump as described previously.7 A number of FHCs were kept frozen in DMEM for control purposes. At the end of the experiments the granuloma or non-wrapped cartilages were carefully dissected free from surrounding tissue. Femoral head cartilages were processed for glycosaminoglycan (GAG) analysis and the results are expressed as the percentage loss compared with frozen controls. The granulomas were weighed immediately and after drying for 48 hours at 56°C and the fluid volume and dry tissue mass were calculated.

**Assessment of levels of sex hormones in serum samples**

Levels of oestradiol were assessed using a commercially available radioimmunoassay (Diagnostic Products, Abingdon, United Kingdom). Serum progesterone was determined by radioimmunoassay as supplied by the North East Thames radioimmunoassay (NETRIA) unit. Testosterone was assessed by an inhouse radioimmunoassay as described previously.8 Interassay coefficients of variation for each steroid were less than 10% at three appropriate concentrations. The same testosterone assay was used to assess androgen levels after implantation of dihydrotestosterone capsules given that the assay shows 80% cross reactivity between the two steroids.

**Hormone treatment and replacement**

In all instances hormonal treatment, when used, was started immediately after the castration or sham operation. Control animals received the appropriate vehicle. In preliminary experiments oestradiol levels were shown to be below the detection limit of our assay (20 pmol/l) in all male animals and during most of the oestrus cycle in female animals. Treatment with this hormone was given by a biweekly subcutaneous injection of 0.5 μg 17β oestradiol in 0.05 ml maize oil in the scruff of the neck. This was the lowest dose capable of inducing oestrous changes in vaginal smears of castrated female animals, the cyclical effects persisting effectively for at least five weeks (data not shown).

Progestosterone was given in a subcutaneous silastic capsule implanted in the ventral surface of the animal. These were prepared using silastic tubing (inner diameter 1-6 mm; outer diameter 2-4 mm; Dow Corning, United Kingdom) sealed at both ends with silastic adhesive as to leave 15 mm free, packed with 15-17 mg of crystalline progesterone. This design was chosen from several tested and shown to replace physiological female levels (80–120 nmol/l) when implanted in castrated female animals, the concentration being stable between the second and fifth week after implantation.

Different designs of testosterone silastic capsules were tested. Figure 1 shows that testosterone capsules replacing physiological levels of this steroid resulted in unacceptably high levels of oestradiol, five weeks after implantation and were abandoned. Dihydrotestosterone subcutaneous silastic capsules (inner diameter 3-4 mm; outer diameter 4-6 mm; 20 mm free) packed with 70–75 mg crystalline hormone resulted in stable androgen levels in the lower physiological range (allowing for the higher potency of dihydrotestosterone) with no detectable increase in oestradiol levels. Dihydrotestosterone was therefore used for androgen replacement.

**IN VITRO CULTURES**

**Ex vivo interleukin 1 production by granulomatous tissue**

Granulomatous tissue surrounding wrapped FHCs was carefully dissected out in a laminar flow hood 10 days after implantation and cultured in 2 ml serum free DMEM for 48 hours. The supernatant was centrifuged, stored at −70°C, and interleukin 1 (IL-1) determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit for mouse IL-1α (Genzyme, United Kingdom), following the manufacturer’s instructions. Interleukin 1 release was corrected for the granuloma dry weight.

**Cartilage culture**

Femoral head cartilages were cultured individually for 10 days in 2 ml serum free and phenol red free DMEM supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, and incubated at 37°C in a
Effect of androgens on cartilage degradation

Assessment of cartilage and medium glycosaminoglycan
Followed implant or culture, FHCs were repeatedly washed and digested overnight at 56°C in 50 mM phosphate buffer (pH 7.0) containing 2 mM N-acetyl cysteine, 1 mM EDTA, and 6 U/ml papain. The GAG content of cartilage digests and culture supernatants was determined following the method of Fardnale et al adapted to allow the use of a plate reader. Glycosaminoglycan loss from implanted FHCs was subsequently expressed as a percentage of the mean of frozen control cartilages. The percentage degradation of cultured FHCs was calculated according to the formula: (GAG medium/(GAG medium+GAG FHC))×100.

Assessment of proteoglycan synthesis in culture cartilage
Pulsed FHCs were vigorously washed in cold 10 mM MgSO4 and digested in papain as described earlier. Aliquots of the digest were precipitated onto filter paper with 1% cetylpyridinium chloride and radioactivity in the precipitate was measured by liquid scintillation counting. Results are expressed in counts per minute (cpm) per milligram of cartilage weight after blot drying.

Statistical methods
Results are expressed as mean (SEM) values. Statistical significance was evaluated by the Mann-Whitney U test for less than 30 samples and Student’s t test for larger numbers. Values of \( p<0.05 \) (two tailed) were taken as significant.

Results
EFFECTS OF COTTON PELLET GRANULOMA ON CARTILAGE DEGRADATION
Groups of intact mice with a double implant of wrapped and non-wrapped FHC were killed at weekly intervals after implantation. Figure 2 shows that GAG loss increased progressively after the first week and the presence of cotton induced granulomatosus tissue resulted in significant acceleration of cartilage destruction. Compared with frozen controls the GAG content of wrapped implanted cartilage showed a significant decrease from week 2 onwards, whereas this only barely achieved statistical significance for four weeks for non-wrapped cartilage. There was a moderate increase in the GAG content at week 1 which was significant for non-wrapped FHC (\( p<0.01 \)). All subsequent experiments were stopped three weeks after implantation.

EFFECTS OF ORCHIDECTOMY ON CARTILAGE DEGRADATION IN THE PRESENCE AND ABSENCE OF GRANULOMA
In the absence of cotton induced granuloma, implanted cartilage lost less than 20% of the initial GAG content and this was not significantly changed by castration (fig 3). In the

\[ \text{Glycosaminoglycan content} \times 100 \]

\[ \text{GAG content of frozen cartilage} \]

\[ \text{GAG content of cartilage} \]

\[ \text{Statistical significance was evaluated by the Mann-Whitney U test for less than 30 samples and Student's t test for larger numbers. Values of } p<0.05 \text{ (two tailed) were taken as significant.} \]
presence of granuloma, however, orchidectomy resulted in significantly accelerated cartilage degradation compared with sham operated animals (59 (2:2) v 42 (2:2); p<0.001). Bilateral cotton wrapped cartilage implants were used in subsequent experiments.

**EFFECTS OF HORMONE TREATMENT AND REPLACEMENT ON CARTILAGE DEGRADATION**

Figure 4 shows the effect on GAG loss of dihydrotestosterone administration to sham operated and castrated male animals implanted with wrapped cartilages. Hormone replacement in castrated male animals resulted in a significant reduction of the rate of GAG loss from implanted cartilages (36:7 (2:7)%) compared with castrated controls (49:8 (3:4%); p<0.01) with complete reversal of the deleterious effects of castration. Treatment of sham operated animals did not confer further protection.

Figures 5 and 6 show the results of treatment of sham operated male animals and male animals which had undergone orchidectomy with oestradiol and progesterone. Oestradiol significantly increased GAG loss in animals which had had an orchidectomy (65:8 (3:5) v 57:7 (1:6)% loss; p<0.05). Treatment with progesterone did not induce significant changes in the rate of cartilage degradation, either in sham operated or castrated male animals, though there was a tendency to accelerate GAG loss in the former group. Combined treatment with oestradiol and progesterone did not result in significant changes compared with control animals (data not shown).

**EFFECTS OF ORCHIDECTOMY ON GRANULOMA FORMATION**

To assess the effects of orchidectomy on the development of granulomatous tissue, the fluid content and dry tissue mass of three week granulomas from animals which had had an orchidectomy and sham operated controls from five different experiments were examined together (table 1). No significant effects of castration were seen in either parameter. Results were similar in each individual experiment and treatment with the various sex hormones did not change granuloma formation (data not shown).

**EX VIVO PRODUCTION OF INTERLEUKIN 1 BY GRANULOMATOUS TISSUE**

Granulomatous tissue dissected 10 days after implantation and cultured for 48 hours released measurable amounts of IL-1α. The total amount released into the supernatant was

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fluid (mg)</th>
<th>Dry mass (mg)</th>
</tr>
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<tbody>
<tr>
<td>Sham operation</td>
<td>16.73 (1.4)</td>
<td>6.94 (0.21)</td>
</tr>
<tr>
<td>Orchidectomy</td>
<td>40.2 (1.6)</td>
<td>7.12 (0.22)</td>
</tr>
</tbody>
</table>

Table 1. Effects of orchidectomy on fluid volume and dry tissue mass of cotton induced granulomas, three weeks after implantation. Results (mean (SEM)) from 40–42 individual animals in control sham and orchidectomised groups from five different experiments were analysed together. None of the differences reached statistical significance (Student’s t test).
significantly higher for granulomas from male animals which had had an orchidectomy (1.3-8 (1.21) pg/mg dry mass; nine animals) compared with those from eight sham operated animals (8.3 (0.65); p=0.002).

IN VITRO EFFECTS OF SEX HORMONES ON CARTILAGE PROTEOGLYCAN SYNTHESIS AND DEGRADATION

Table 2 shows that all three hormones (testosterone, oestradiol, and progesterone) induced a significant reduction of the rate of spontaneous GAG release into the supernatant when tested in physiological concentrations for male rats. Effects were more marked with progesterone, which, at a concentration of 1 mmol/l, reduced GAG release from 27.1% in controls to 20.7% (p<0.001). These effects were not dose dependent. None of the sex hormones influenced significantly the rate of proteoglycan synthesis as measured by incorporation of sulphur-35.

IN VITRO INTERACTION OF SEX HORMONES WITH INTERLEUKIN 1α ON CARTILAGE PROTEOGLYCAN SYNTHESIS AND DEGRADATION

The addition of 1 ng/ml mrIL-1α during the second five day culture period resulted in a significant increase of cartilage degradation (3.76 v 6.52×10^3 cpm/ mg; p<0.001). The effects of IL-1 on the two parameters were not significantly improved by preincubation and the continuous presence of sex hormones (table 3).

**Discussion**

Wrapping cartilage in cotton induces the formation of granulomatous tissue and a marked increase in cartilage damage, providing a suitable model to assess the effects of proteoglycan synthesis above the negative effects of early inflammation. Differences between wrapped and nonwrapped cartilages were not statistically significant and although they may have contributed to the differences seen at later points, these are much greater and increase with the time of implantation. Orchidectomy consistently resulted in an accelerated rate of cartilage loss in the presence of granulomatous reaction, coinciding with an increased production of IL-1. The effects of orchidectomy were completely nullified by the replacement of physiological levels of dihydrotestosterone, thus confirming the protective role of androgens in this process. The lack of further protection when dihydrotestosterone was given to these hormone treated animals shows that the maximum androgen mediated effects in this model were achieved at physiological levels.

Progestosterone did not have any significant effect but oestradiol caused a significant increase in cartilage loss in animals which had had an orchidectomy but not in sham operated male animals, suggesting that androgens may counteract the deleterious effects of oestradiol. The levels of progesterone, and presumably oestradiol, achieved by our treatment regimens were supraphysiological for male mice, but our results nevertheless show that these hormones do not offer protection against cartilage degradation in the male animal, despite their known immunosuppressive actions. These results corroborate observations of increased susceptibility and severity of streptococcal cell wall arthritis after castration or oestrogen treatment in male LEW/N rats.10

Investigations of the effects of sex hormones in inflammatory disorders face a number of methodological difficulties derived mostly from the complexity of hormone regulation and metabolism and the intricate interactions between the gonadal, adrenal, and immune systems.4 11 In vivo models allow the assessment of end results that cannot be directly predicted from simplified in vitro systems. The model we used is not intended as a direct reproduction of RA but as a model of cartilage damage induced by granulomatous tissue with similar features to rheumatoid pannus. Its features have been well described and published results on the effects of current treatments for
rheumatic diseases have shown a good correlation with experience in human disease and other experimental models. Unlike more conventional models it allows a reproducible measurement of cartilage destruction offering appropriate conditions for the study of this essential component of arthritis.

The peripheral transformation of testosterone into oestradiol has been shown to be an important confounding factor on studies addressing immunological effects of sex hormones. Our results show that prolonged testosterone treatment of experimental male animals, in a dose sufficient to attain physiological levels, will result in extremely high oestradiol levels due to peripheral transformation and is, therefore, inadequate to study the effects of androgens. The silastic capsules of 5α-dihydrotestosterone used here allowed stable androgen levels to be obtained in the physiological range (compensating for the higher androgenic activity of dihydrotestosterone) without any detectable change in oestradiol levels.

Unsuspected sex hormone action is also a potential problem in in vitro studies. We have repeatedly found high levels of oestradiol and progesterone in different batches of fetal calf serum. Progesterone is also a common component of most available serum alternatives for culture purposes. Weak oestrogenic properties of phenol red, a common pH indicator in culture media, have been shown repeatedly and we have therefore avoided these components in our culture conditions.

The mechanisms of the protective actions of androgens are still unclear. The lack of influence of orchidectomy on non-wrapped cartilage suggests that the effects of sex hormones are mainly due to actions on the inflammatory tissue and not directly on the chondrocytes. To clarify this point further we investigated the effects of sex steroids and their interaction with IL-1 on cartilage metabolism in vitro. The effects seen on spontaneous proteoglycan loss in culture are common to the different sex steroids studied and do not seem to be important in vivo. None of the sex hormones showed any significant interference with IL-1 induced effects on proteoglycan synthesis and degradation in vitro, reinforcing the concept that direct actions on cartilage do not contribute significantly to the overall effects seen in the in vivo model. The lack of dose dependency of sex hormone effects on cartilage is probably related to the number and kinetics of the respective receptors in this tissue. To our knowledge only oestradiol receptors have been described in articular chondrocytes. These receptors were found in small amounts but showed high affinity, which may justify saturation at physiological concentrations. It should also be noted that the lower concentrations used in our in vitro studies correspond approximately to physiological concentrations of free sex steroids, given that these hormones are bound to carrier proteins in excess of 90%.

Orchidectomy did not affect parameters of granulomatous tissue development, in agree-
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