

INFLAMMATION-INDUCED CARTILAGE DEGRADATION IN FEMALE RODENTS

Protective Role of Sex Hormones

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Objective. To investigate the effects of physiologic levels of sex steroids on inflammation and cytokine production and their consequential cartilage degradation.

Methods. We used an *in vivo* model of inflammation-induced cartilage degradation in female mice to study the effects of ovariectomy and hormone treatment, and *in vitro* culture systems to examine the influence of sex steroids on cartilage metabolism, interleukin-1 (IL-1) production by granulomatous tissue, and its effects on female mouse articular cartilage.

Results. Ovariectomy resulted in accelerated cartilage breakdown associated with increased production of IL-1 by granulomatous tissue. The effects of ovariectomy on cartilage were reversed by treatment with estradiol and androgen, but not by progesterone treatment. Estradiol and progesterone reduced both sponta-

neous and IL-1-induced cartilage degradation *in vitro*. Testosterone antagonized the effects of IL-1 on both proteoglycan loss and proteoglycan synthesis.

Conclusion. These data suggest that sex steroids have an important influence on inflammation-induced cartilage breakdown in female animals, with protective effects of both estradiol and androgens. Multiple mechanisms may be involved, and they are likely to include direct immunomodulatory effects as well as interactions with the effects of cytokine and of the glucocorticoid response to inflammation.

Rheumatoid arthritis (RA) is 2–3 times more frequent in females than in males. In addition, in females it tends to have a more aggressive and disabling course, despite an apparently balanced distribution of other risk and prognostic factors such as positivity for HLA-DR4 and for rheumatoid factor. Further evidence for an important pathogenic role of sex hormones in RA comes from the increased rate of onset during the reproductive years, changes with the menstrual cycle, and beneficial effects of oral contraceptives on the incidence and severity of the disease. Work with experimental models has demonstrated that ovariectomy increases the susceptibility to and severity of both collagen-induced and adjuvant-induced arthritis in female rodents, and that this is reversed by replacement of estradiol at physiologic levels (for review, see ref. 1). Moreover, maintenance of gestational levels of estradiol has been shown to prevent the postpartum flare that follows pregnancy-associated remission of collagen-induced arthritis (2).

Immunologic effects of sex hormones are well recognized and are thought to underlie their actions in human and experimental disease (3). Overall, estro-

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gens stimulate B cell-mediated, and depress T cell-mediated, immune responses, while progesterone and androgens show immunosuppressive properties over both. Such observations have prompted a number of studies on the therapeutic potential of estrogens in human RA, with generally disappointing results. However, most published studies have been of short duration (≤ 6 months) and have focused on parameters of disease activity rather than disease progression. The use of synthetic hormones, with varying immunosuppressive properties, in these studies may also contribute to the negative results. Recently, intraarticular progesterone was found to suppress inflammation in rheumatoid joints (4), while testosterone replacement had beneficial effects on disease activity in hypogonadic male patients (5).

Cartilage degradation is a major consequence of RA and plays a determinant role in joint destruction and disability. In the present study, using a model of inflammation-induced cartilage degradation and *in vitro* cultures of cartilage and granulomatous tissue, we assessed the effects of sex steroids and their interaction with inflammation on cartilage loss in arthritis.

MATERIALS AND METHODS

Ovariectomy and sham operation. Mature (9–10-week-old) female BALB/c mice (Tuck & Sons, Battlesbridge, UK) were used. Experimental and control (sham-operated) animals were age matched in all experiments. The mice were kept under controlled light conditions (14 hours light/10 hours dark). All surgical procedures were performed under intraperitoneally administered anesthesia (0.07 ml of combined Hypnorm [Janssen, Pettebridge, UK] and Hypnovel [Roche, Welwyn Garden City, UK] in water [1:1:2 volume/volume/volume]). Ovariectomy was performed through a single dorsal midline cutaneous incision followed by bilateral muscle incisions. Sham-operated animals underwent a similar procedure but the gonads were replaced in the abdomen after exposure. Hormone treatment and replacement was started at this time, as described below. Further surgical procedures were performed after a 2-week recovery period. Sexual maturity was ascertained by visual inspection of the gonads, and efficiency of castration was verified by sequential vaginal smear examination.

Preparation of cartilage. Female Wistar rats weighing 130–150 gm (Tuck & Sons) were killed and both femoral head cartilages (FHC) were dissected free of bone and pooled in Hanks' balanced salt solution (HBSS) containing 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cartilage samples were washed thoroughly in HBSS, trimmed free of adherent tissue under aseptic conditions, and kept 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).

Cartilage implants. FHC were aseptically prepared for implantation by wrapping in sterile surgical cotton gauze (Softsorb, mean \pm SEM weight 5 ± 0.15 mg; Vernon-Carus, Preston, UK) moisturized with HBSS. Each mouse received a bilateral subcutaneous implant of either wrapped or non-wrapped FHC at each side of the dorsal hump, as previously described (6). A number of FHC samples were kept frozen in DMEM for control purposes. At the end of the experiments, granulomas or nonwrapped cartilages were carefully dissected free from surrounding tissue. FHC were processed for glycosaminoglycan analysis as described below. The granulomas were weighed immediately and after drying for 48 hours at 56°C, and the fluid volume and dry tissue mass were calculated. Potential interference of the inflammatory process in the normal estrus cycle was assessed by examination of vaginal smears from sham-operated controls.

Hormone treatment and replacement. In all cases, hormone treatment, when used, was started immediately after castration/sham-operation and continued throughout the experiment. Control animals received the appropriate vehicle. In preliminary experiments, estradiol levels were shown to be below the detection limit of our assay (20 pmoles/liter) in all phases of the estrus cycle. Treatment with this hormone was administered by a biweekly subcutaneous injection of 0.5 μg 17 β -estradiol in 0.05 ml of maize oil, in the scruff of the neck. This was the lowest dose able to induce estrus changes in vaginal smears of castrated females, with cyclical effects that persisted effectively for the 5 weeks of treatment in experimental animals (data not shown).

Progesterone was given in a subcutaneous silastic capsule implanted in the ventral surface of the animal. These were prepared using silastic tubing (inner diameter 0.062", outer diameter 0.095"; Dow Corning, Reading, UK) sealed at both ends with silastic adhesive so as to leave 15 mm free, left empty or packed with 15–17 mg of crystalline progesterone. This particular design was shown to replace progesterone at physiologic levels (80–120 nmoles/liter) when implanted into castrated females, the concentration being stable between the second and fifth week postimplantation.

For androgen replacement/treatment, dihydrotestosterone (DHT) silastic capsules (inner diameter 0.132", outer diameter 0.183", 20 mm free), empty or packed with 70–75 mg of crystalline hormone, were used. These were shown to replace androgens at near physiologic levels when implanted into castrated males, with no detectable increase in estradiol levels. All hormones were obtained from Sigma (Poole, UK).

Cartilage cultures. FHC samples, prepared as above, were cultured individually for 5 days in 2 ml of serum-free DMEM without phenol red, supplemented with antibiotics, and incubated as above. Sex hormones were added to the medium from stock solutions in DMSO, to final concentrations selected to cover a range from 10-fold below to 10-fold above average physiologic levels in mature female Wistar rats as determined in preliminary experiments. The final concentration of DMSO was 0.01% (v/v) in all groups.

In the experiments using interleukin-1 (IL-1), cultures were extended for 10 days. Medium was changed at 5 days and recombinant mouse IL-1 α (Genzyme, West

Malling, UK), prepared in DMEM with 0.5% bovine serum albumin (BSA), was added to the medium in the second 5-day period, to a final concentration of 1 ng/ml. A similar volume of 0.5% BSA was added to control wells.

Measurement of ex vivo IL-1 production by granulomatous tissue. Ten days after implantation, granulomatous tissue surrounding wrapped FHC was carefully dissected out in a laminar flow hood, and cultured in 2 ml of serum-free DMEM for 48 hours. Supernatants were centrifuged and stored at -70°C until analysis. The weight of each granuloma was recorded after drying for 48 hours at 56°C . The concentration of IL-1 in the supernatant was assessed with a commercially available enzyme-linked immunosorbent assay kit for mouse IL-1 α (Genzyme), according to the manufacturer's instructions, and the results corrected for granuloma dry weight.

Assessment of cartilage and medium glycosaminoglycan (GAG). Following implant or culture, FHCs were repeatedly washed and digested overnight at 56°C , in 50 mM phosphate buffer (pH 7.0) containing 0.33 gm/liter *N*-acetylcysteine, 1 mM EDTA, and 6 units/ml papain. The GAG content of cartilage digests and medium supernatants was determined according to the method of Farndale et al (7), adapted to allow the use of a plate reader. GAG loss from implanted FHC was subsequently expressed as a percentage of the mean in frozen control cartilages. Percentage degradation of cultured FHCs was calculated according to the formula

$$\frac{\text{GAG medium}}{\text{GAG medium} + \text{GAG FHC}} \times 100$$

Assessment of proteoglycan synthesis in cultured cartilage. Cultured FHCs were pulsed with 1 $\mu\text{Ci/ml}$ carrier-free $\text{Na}_2^{35}\text{SO}_4$ (Amersham, Amersham, UK) for the last 24 hours of culture. FHCs were vigorously washed in cold MgSO_4 and digested in papain as above. Aliquots of the digest were precipitated onto filter paper with 1% cetylpyridinium chloride, and radioactivity in the precipitate was measured by liquid scintillation. Results were expressed in counts per minute $\times 10^{-3}$ per mg of cartilage wet weight.

Statistical analysis. All results are presented as the mean \pm SEM. The Mann-Whitney U test was used when *n* values were less than 30, and Student's *t*-test for larger numbers. *P* values less than 0.05 (2-tailed) were considered significant.

RESULTS

Effects of ovariectomy on cartilage degradation, in the presence and absence of granuloma. The presence of cotton-induced granuloma resulted in a significantly increased rate of cartilage destruction in both sham-operated and ovariectomized female animals ($P < 0.001$) (Figure 1). In the absence of cotton-induced granuloma, implanted cartilage lost up to 30% of its initial GAG content 3 weeks after implantation, and this was not significantly changed by castration. In the presence of granuloma, however, ovariectomy

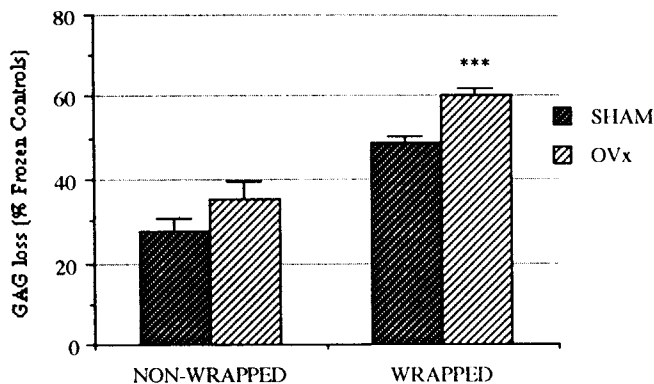


Figure 1. Effects of ovariectomy (OVx) on glycosaminoglycan (GAG) loss from implanted cartilage. Female mice, either sham operated or ovariectomized 2 weeks previously, received implants of either cotton-wrapped or nonwrapped cartilage. Experiments were terminated at 3 weeks after implantation. Values are the mean and SEM from 5 different experiments for wrapped cartilage ($n = 40-41$) and 2 experiments for nonwrapped cartilage ($n = 14$). *** = $P < 0.001$ versus sham-operated animals, by Student's *t*-test.

significantly accelerated cartilage degradation in comparison with that in sham-operated animals ($60 \pm 1.7\%$ versus $48.7 \pm 1.5\%$; $P < 0.001$). Vaginal smears of control sham-operated females examined throughout the full duration of several experiments showed a regular estrus cycle of 4-5 days, similar to that observed in the absence of implants.

Effects of hormone treatment and replacement on cartilage degradation. The results of treatment of sham-operated and ovariectomized females with estradiol, progesterone, and DHT are shown in Figure 2. Estradiol replacement resulted in significant protection of cartilage in ovariectomized females, with complete abrogation of the effects of gonadectomy. Sham-operated animals treated with estradiol had a reduction in the rate of GAG loss ($32 \pm 3.5\%$, versus $43.2 \pm 3.5\%$ in sham-operated controls), although this just failed to reach significance ($P = 0.054$). Progesterone administration did not affect the rate of cartilage degradation in either sham-operated or ovariectomized females. Combined treatment with estradiol and progesterone resulted in changes similar to the ones obtained using estradiol alone, with no evidence of potentiation or antagonistic effects.

Treatment with DHT did not change the rate of cartilage destruction in intact females but significantly reduced it in ovariectomized animals ($40.7 \pm 3.1\%$ versus $54.9 \pm 2.7\%$; $P < 0.01$). Sham-operated controls showed a significantly greater amount of cartilage de-

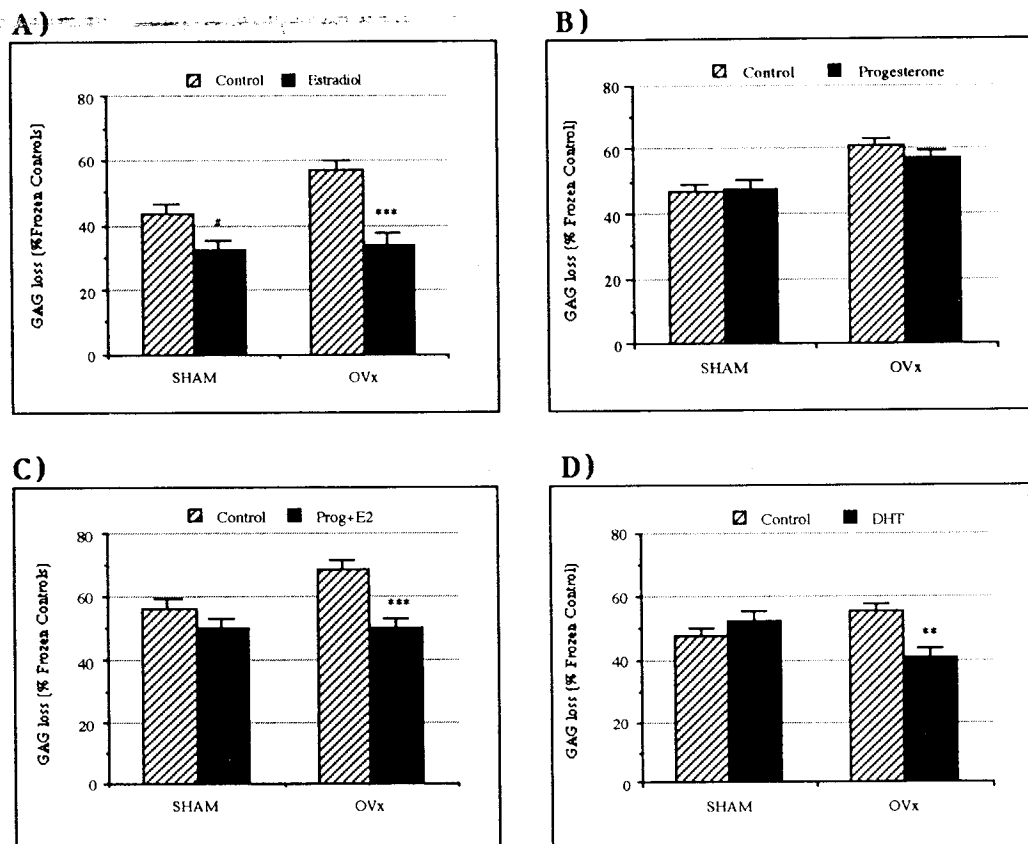


Figure 2. Effects of hormone treatment on glycosaminoglycan (GAG) loss from implanted cotton-wrapped cartilage. Female mice underwent either ovariectomy (OVx) or sham operation, after which estradiol (A), progesterone (B), progesterone plus estradiol (Prog+E2) (C), or dihydrotestosterone (DHT) (D) treatment was immediately started and continued for 5 weeks. Control animals received the appropriate vehicle. Two weeks after ovariectomy or sham operation, each animal received a subcutaneous implant of cotton-wrapped cartilage. Experiments were terminated at 3 weeks after implantation. # = $P = 0.054$ versus sham-operated controls; *** = $P < 0.001$ versus ovariectomized controls; ** = $P < 0.01$ versus ovariectomized controls, by Mann-Whitney U test ($n = 9-10$ for all groups).

gradation than did ovariectomized animals treated with DHT ($47.8 \pm 1.9\%$ versus $40.7 \pm 3.14\%$; $P < 0.05$).

Ex vivo production of IL-1 by granulomatous tissue. Granulomatous tissue dissected at 10 days postimplantation and cultured for 48 hours produced measurable amounts of IL-1 α . The total amount released into the supernatant, corrected for dry tissue mass, was significantly higher in granulomas from ovariectomized females (21.1 ± 3.5 pg/mg dry mass; $n = 9$) in comparison with those from sham-operated animals (9.5 ± 1.6 pg/mg; $n = 8$) ($P = 0.012$).

In vitro effects of sex hormones on cartilage proteoglycan synthesis and degradation. The results of studies of sex hormone effects on proteoglycan synthesis and degradation by articular cartilage in vitro

are shown in Table 1. Both estradiol and progesterone induced a dose-dependent reduction of spontaneous GAG loss, the differences being significant at 1 nM estradiol and 50 nM progesterone. Testosterone was devoid of significant effects. None of the hormones significantly changed the rate of proteoglycan synthesis as measured by ^{35}S incorporation.

Effects of the in vitro interaction of sex hormones and IL-1 α on cartilage proteoglycan synthesis and degradation. Recombinant mouse IL-1 α , in a concentration of 1 ng/ml, increased spontaneous GAG loss and reduced proteoglycan synthesis by cultured rat cartilages (Table 2). Both progesterone and testosterone antagonized the effects of IL-1 on GAG loss in a dose-dependent manner; the differences were signifi-

Table 1. Effects of sex hormones on proteoglycan loss and synthesis by cultured cartilage*

Treatment, hormone concentration	% degradation	³⁵ S incorporation (cpm × 10 ⁻³ /mg)
DMEM/DMSO alone	25.0 ± 1.25	3.15 ± 0.12
DMEM/DMSO + E2		
10 ⁻¹¹ M	26.7 ± 1.06	3.13 ± 0.19
10 ⁻¹⁰ M†	23.4 ± 0.78	3.20 ± 0.19
10 ⁻⁹ M	20.0 ± 1.52‡	3.00 ± 0.20
DMEM/DMSO + Pg		
5 × 10 ⁻⁹ M	25.7 ± 1.4	3.45 ± 0.18
5 × 10 ⁻⁸ M†	21.0 ± 1.1‡	3.38 ± 0.21
5 × 10 ⁻⁷ M	20.4 ± 1.6§	2.84 ± 0.17
DMEM/DMSO + T		
10 ⁻¹⁰ M	24.1 ± 1.33	3.07 ± 0.17
10 ⁻⁹ M†	25.6 ± 1.35	3.50 ± 0.17
10 ⁻⁸ M	24.5 ± 0.73	3.35 ± 0.13

* Rat femoral head cartilages were cultured for 5 days in the presence of various concentrations of sex hormones or vehicles alone, and pulsed with ³⁵S for the last 24 hours. Values are the mean ± SEM (n = 8 for all groups). DMEM = Dulbecco's modified Eagle's medium; E2 = estradiol; Pg = progesterone; T = testosterone.

† Average physiologic level in mature female Wistar rats.

‡ P < 0.05 versus DMEM/DMSO alone, by Mann-Whitney U test.

§ P = 0.054 versus DMEM/DMSO alone, by Mann-Whitney U test.

cant at physiologic levels and at concentrations 10-fold higher. Estradiol inhibited IL-1-induced cartilage degradation at a concentration of 10 pM.

Treatment with testosterone, at a concentration of 10 nM, resulted in complete inhibition of the IL-1-induced decrease in proteoglycan synthesis, while neither estradiol nor progesterone had such an effect. Overall, the combined effects of these steroids resulted in a net gain of GAG content (gain of 23.8 ± 0.77 μg/mg wet weight for cartilages treated with 1 nM estradiol and 25.6 ± 0.84 μg/mg for those treated with 10 nM testosterone; P < 0.05 and P < 0.01, respectively, versus cartilages treated with IL-1 alone [20.4 ± 1.53 μg/mg]).

DISCUSSION

The occurrence of a granulomatous reaction in the vicinity of cartilage resulted in a marked acceleration of the rate of cartilage degradation, consistent with the concept that active synovitis is the driving force behind progressive joint destruction in inflammatory arthritis (8). Ovariectomy consistently resulted in marked increases of inflammation-induced GAG loss, correlating with higher production of IL-1 by granulomatous tissue, and thus indicating an important modulatory role for sex hormones in this process. The

results obtained with hormone treatment established that estradiol plays a critical protective role: Replacement of physiologic levels of estradiol in ovariectomized females abrogated the deleterious effects of ovariectomy, while treatment with progesterone did not result in significant changes. This is consistent with observations in both collagen-induced and adjuvant-induced arthritis, although we failed to show any potentiation of the effects of estradiol by progesterone as seen with these other models. Cartilage degradation was reduced by androgen treatment in ovariectomized, but not in sham-operated, females. Despite the difficulties in transposing findings of animal studies to human conditions, these results corroborate observations of deleterious effects of aging and the beneficial actions of contraceptive and noncontraceptive hormone treatment in human RA.

The model we used is not intended as a direct reproduction of RA, but as a system of cartilage degradation by juxtaposed granulomatous tissue similar to the situation in rheumatoid joints. Unlike conventional models, it allows reproducible quantitation of end effects on cartilage integrity, a process of major importance in arthritis progression and consequent disability. The development of cotton-induced granu-

Table 2. Effects of the interaction of sex hormones and interleukin-1 (IL-1) on cartilage proteoglycan loss and synthesis*

Treatment, hormone concentration	% degradation	³⁵ S incorporation (cpm × 10 ⁻³ /mg)
DMEM/DMSO alone	16.6 ± 1.22	3.71 ± 0.12
IL-1α/DMSO alone	22.4 ± 1.17†	2.70 ± 0.23‡
IL-1α/DMSO + E2		
10 ⁻¹¹ M	18.3 ± 1.0§	2.64 ± 0.16
10 ⁻¹⁰ M¶	20.7 ± 0.74	2.75 ± 0.21
10 ⁻⁹ M	20.2 ± 0.83	3.11 ± 0.22
IL-1α/DMSO + Pg		
5 × 10 ⁻⁹ M	20.5 ± 1.93	2.62 ± 0.14
5 × 10 ⁻⁸ M¶	18.3 ± 0.9§	2.63 ± 0.24
5 × 10 ⁻⁷ M	15.4 ± 1.3#	3.26 ± 0.20
IL-1α/DMSO + T		
10 ⁻¹⁰ M	23.2 ± 1.42	2.83 ± 0.35
10 ⁻⁹ M¶	18.4 ± 1.1§	2.96 ± 0.23
10 ⁻⁸ M	17.8 ± 0.64#	3.68 ± 0.16#

* Rat femoral head cartilages were cultured for 10 days in the presence of sex steroids, with one change of medium at day 5. Mouse recombinant IL-1α (1 ng/ml) was added to the medium for the second culture period, and cartilages were pulsed with ³⁵S for the last 24 hours. Values are the mean ± SEM (n = 8 for all groups). See Table 1 for other definitions.

† P < 0.01 versus DMEM/DMSO alone.

‡ P < 0.001 versus DMEM/DMSO alone.

§ P < 0.05 versus IL-1α/DMSO alone.

¶ Average physiologic level in mature female Wistar rats.

P < 0.01 versus IL-1α/DMSO alone.

granulomatous tissue is a T cell-dependent process (9), and mature tissue is predominantly composed of fibroblasts, mononuclear cells, polymorphonuclear cells (PMNs), and some giant cells (6). Earlier work by our group has demonstrated that immune response to components of the implanted cartilage plays a role in the degradation, since previous exposure to cartilage implants resulted in increased degradation in a subsequent challenge. This potentiation was more pronounced when the first implant included cotton, suggesting that the granulomatous tissue acts as an adjuvant to increase the immunogenicity of the implanted cartilage (10).

The exact mechanism of the immune response to cartilage has not been elucidated. Recent work has demonstrated the direct involvement of metalloproteases in cartilage damage in vivo (11), and our own results demonstrate the production of significant amounts of IL-1, capable of inducing chondrocyte-mediated matrix loss.

Sex hormones may interfere with this system at multiple levels, i.e., through direct effects on cartilage, distribution and degranulation of PMNs (12), modulation of T cell-mediated responses (3), and cytokine production (13), among others. Direct effects on cartilage would seem to be of minor importance in the in vivo model given that ovariectomy did not influence GAG loss in the absence of inflammation. The possibility cannot be excluded, however, that sex steroids exert significant influence on cartilage response to the granulomatous aggression. We have observed a significant reduction of spontaneous proteoglycan loss from cartilage in vitro with periphysiologic concentrations of estradiol and progesterone. Furthermore, periphysiologic concentrations of all 3 sex steroids tested were able to inhibit IL-1-induced GAG loss, and testosterone also antagonized its effects upon proteoglycan synthesis. Such effects may have an important contribution to cartilage protection over longer periods of time.

Ovariectomy did not significantly change the fluid content or dry mass of inflammatory tissue. However, the increased production of IL-1 by granulomas from ovariectomized females is likely to play an important role in the effects of castration on cartilage breakdown. Such differences may be due to direct effects of sex steroids, since female sex hormones are known to directly influence IL-1 production by human and murine peripheral monocytes and tissue macrophages (13,14). Links between these observations and human disease are strengthened by the finding of

elevated peripheral monocyte IL-1 secretion, which is normalized by estrogen/progestogen replacement, in postmenopausal women (15).

Direct antiinflammatory actions of sex steroids may also play a role. Cartilage protection and reduction of cell infiltration with intralesional testosterone have been described in models of cotton pellet-induced and antigen-induced arthritis in rats (16), and antiinflammatory effects of estrogens have been demonstrated in various models of inflammation (17).

The interactions between the gonadal and the adrenal axis may offer additional contributions. Compared with male animals, female rodents are known to present higher levels of circulating corticosterone both at baseline and in response to a variety of stimuli, and these levels are reduced by castration and restored by estradiol. Using the present model, we have been able to show similar sex differences and effects of female sex hormones in response to granulomatous inflammation and IL-1 (18). This would suggest that the interaction with the hypothalamic-pituitary-adrenal (HPA) axis may contribute significantly to the effects of estradiol and castration in different animal models. However, the beneficial effects of DHT in ovariectomized females appear to be independent of the HPA axis, since we have observed that androgens effectively reduce the glucocorticoid response to inflammation (18).

In summary, our results show that reduction of female sex hormones may result in accelerated cartilage degradation in an inflammatory context, an effect that is reversed by replacement of estradiol at physiologic levels and by therapeutic doses of androgens. Direct or indirect modulation of IL-1 production by the inflammatory tissue and protection of cartilage against IL-1-induced degradation may contribute significantly to these effects. We suggest that interactions with the HPA axis play an important role in the antiinflammatory and immunosuppressive effects of estradiol in vivo. If similar mechanisms are shown to occur in human disease, our understanding of the role of sex steroids and glucocorticoids in the pathogenesis and therapy of inflammatory and autoimmune diseases will be greatly improved.

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