EXTENDED REPORT

Dual effects of 17 β -oestradiol on interleukin 1 β -induced proteoglycan degradation in chondrocytes

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Objective: To determine whether 17 β -oestradiol (E2) modulates interleukin (IL) 1 β -induced proteoglycan degradation in chondrocytes, and to analyse the part played by metalloproteinases (MMPs) in this process.

Methods: Primary cultured rabbit articular chondrocytes were prepared and treated with 10 ng/ml IL1 β combined or not with 0.1–10 nM E2. Neosynthesised proteoglycans (PGs) were evaluated after incorporation of [³⁵SO₄]sulphate and further analysed after chromatography on a Sepharose 2B column. Chondrocyte mRNA levels of aggrecan, MMP-1, -3, -13, and tissue inhibitor of metalloproteinase-1 (TIMP-1) were studied by northern blot. MMP-1 activity was measured by zymography. MMP-1 gene transcription was studied by transient transfection of chondrocytes with an MMP-1-luciferase construct.

Results: E2 modulated the IL1 β -induced total sulphated PGs in rabbit articular chondrocytes, which decreased as the E2 concentration was increased. At a low concentration (0.1 nmol/l) E2 counteracts the IL1 β -induced decrease in sulphated PG, while at high concentration (10 nmol/l) E2 enhances the IL1 β effects. A biphasic E2 effect was also observed on IL1 β -induced disaggregation of PG, 53–58 kDa gelatinolytic activity, and MMP-1, -3, and -13 mRNA levels. In contrast, E2 did not modify the level of aggrecan mRNA and had no effect on TIMP-1 mRNA expression. Finally, simultaneous addition of IL1 β and E2 (0.1–10 nmol/l) did not modify IL1 β -induced MMP-1-luciferase activity, suggesting that E2 effects probably occur at the post-transcriptional level of MMP gene expression.

Conclusion: Oestrogen concentration may have an inverse effect on IL1 β stimulated proteoglycan degradation and MMP production by chondrocytes.

Osteoarthritis (OA) is the most common cause of musculoskeletal pain and disability. It is characterised by cartilage loss, subchondral sclerosis, cyst, and osteophyte formation. Although OA is a progressive and heterogeneous disorder of unknown aetiology, mechanical and genetic factors appear to have a major role.¹

Hormonal changes occurring around the menopause have long been thought to affect the occurrence of OA.¹ Epidemiological studies suggest that oestrogen loss may be accompanied by an increase in the prevalence and incidence of knee and hip OA. In support of a role for oestrogen (17 β -oestradiol (E2)) in cartilage metabolism, other studies have provided some evidence that women taking oestrogen replacement therapy have a lower than expected risk of gonarthrosis or coxarthrosis than controls.²

OA is characterised by a degeneration of articular cartilage, marked by breakdown of matrix proteins. This leads to the development of fibrillations, fissures, and ulcerations at the articular cartilage surface. Cartilage degradation is mediated by metalloproteinases, which specifically cleave matrix proteins.³ Metalloproteinases are classified in two groups: matrix metalloproteinases (MMPs) and a family of metalloproteinases with thrombospondin motifs (ADAMTs) also called aggrecanases.³ There is currently extensive evidence that among the MMPs, MMP-1 (collagenase 1), MMP-3 (stromelysin 1), and MMP-13 (collagenase 3) are particularly involved in the OA process.^{4–5} MMPs and aggrecanases are implicated in the breakdown of collagen and aggrecan, respectively. However, recent data indicated that MMPs may also be implicated in the aggrecan proteolysis during cartilage degradation.^{6–7}

A series of in vivo and in vitro studies have shown that cartilage is a hormone-sensitive tissue and that both isoforms of oestrogen receptor α and β are present in chondrocytes

from human and other species.^{8–10} In vivo animal studies suggest that oestrogen replacement therapy may have a protective effect against OA; it reverses the loss of biomechanical properties of cartilage induced by ovariectomy in sheep¹¹ and reduces the severity of cartilage degradation in ovariectomised monkeys as observed at the histological level.¹²

The mechanism by which oestrogen replacement therapy prevents cartilage degradation may occur via the production of growth factors from the insulin-like growth factor family, which in turn increases matrix protein synthesis by chondrocytes. Ex vivo experiments reported by Richmond *et al*¹⁰ and by Fernihough *et al*¹³ are in accordance with this hypothesis. They observed an increased proteoglycan (PG) synthesis by chondrocytes cultured from ovariectomised monkeys receiving oestrogen replacement therapy in comparison with untreated animals.¹⁰ In vitro studies reported an increased amount of PGs by cultured chondrocytes treated with E2 in comparison with untreated cells.^{13–16} Whether or not the E2-induced augmentation of matrix proteins was due to increased synthesis or decreased degradation of PGs remains unknown.

In favour of the latter hypothesis, experimental data reported on other cell types suggest that E2 modulates MMPs. Rajabi *et al* reported that treatment of guinea pig uterine cervical cells with 10 nM E2 up regulated MMP-1

Abbreviations: AP-1, activated protein-1; CPC, cetylpyridinium chloride; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMW, high molecular weight; IL, interleukin; LMW, low molecular weight; MMP, matrix metalloproteinase; OA, osteoarthritis; PG, proteoglycan; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulphate; TIMP-1, tissue inhibitor of metalloproteinase-1

Table 1 Oligonucleotide primers used for RT-PCR

Gene	Primers	Product size (bp)	Annealing temperature (°C)	Genbank accession number [ref]
MMP-1	5'-TCA GTT CGT CCT CAC TCC AG-3' 5'-TTG GTC CAC CTG TCA TCT TC-3'	322	55	gb M17821 [27]
MMP-3	5'-ATG GAC CTT CTT CAG CAA-3' 5'-TCA TTA TGT CAG CCT CTC-3'	391	54	gb M25664
MMP-13	5'-AGG AGC ATG GCG ACT TCT AC-3' 5'-TAA AAA CAG CTC CGC ATC AA-3'	458	55	gb M002427
Aggrecan	5'-CTC ACC CCG AGA ATC AAA TG-3' 5'-AGG AGG TTT CCG CCG CAG TT-3'	744	59	gb L38480
TIMP-1	5'-GCA ACT CCG ACC TTG TCA TC-3' 5'-AGC GTA GGT CTT GGT GAA GC-3'	326	55	gb J074112 [27]

gb, Genbank accession number.

expression at the mRNA and protein levels.¹⁷ Oestradiol was also shown to up regulate MMP-2 and MMP-9 in mice mesangial cells.^{18,19} By contrast, Liao *et al* recently reported that 0.1–10 nM E2 represses MMP-1 synthesis in human osteosarcoma cells (MG-63) and in human normal osteoblasts in culture.²⁰

In osteoarthritic cartilage, expression of metalloproteinases is strongly stimulated by inflammatory cytokines such as interleukin (IL) 1β.⁴

The purpose of our work was thus to study whether E2 modulates IL1β-induced PG degradation, and to analyse the involvement of MMPs in this process.

MATERIAL AND METHODS

Isolation and culture of chondrocytes from rabbit articular cartilage

Rabbit articular chondrocytes were cultured as previously described.²¹ Briefly, 5 week old Fauve de Bourgogne rabbits (Centre de production animale, Olivet, Orléans, France) were killed and articular cartilages were removed under sterile conditions. Thin slices of cartilage were sequentially digested and the resulting cell suspension was transferred to 75 cm² culture flasks, at 10⁵ cells/cm² (high density) containing 12 ml Ham's F12 medium (Gibco BRL-Life Technologies), 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Roche, Germany) with initial pH 7.2–7.6. Cells were then cultured at 37°C in an atmosphere of 8% CO₂ in air, and the medium was changed once until confluency (day 6 of the culture).

Treatment of chondrocytes with different effectors

At confluency, the medium was replaced with phenol red free Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL-Life Technologies) with or without Na₂[³⁵SO₄] and to which were added either 10 ng/ml IL1β alone (Immugenex Corporation, Los Angeles, USA) or combined with E2 (Sigma-Aldrich, France) at concentrations ranging between 0.1 nmol/l (10⁻¹⁰ mol/l) and 10 nmol/l (10⁻⁸ mol/l). Rabbit articular chondrocytes were incubated for 20 hours and analysed for ³⁵SO₄ labelled PGs or mRNA expression levels of MMPs. Cell culture supernatants were recovered and stored at -80°C for zymography analysis.

Measurement of [³⁵SO₄]sulphate incorporation into PGs

The incorporation of [³⁵SO₄]sulphate into PGs by articular chondrocytes was assessed as previously described.¹⁴ At confluency, chondrocytes were incubated in serum-free, sulphate-free DMEM plus 1.5 μCi/ml Na₂[³⁵SO₄] (75 MBq/ml; Amersham, Buckinghamshire, England) for 20 hours in the presence or absence of effectors. Controls were prepared without any additive compound. Six similarly treated flasks were prepared for each experiment, three of them were used

for ³⁵SO₄ measurement and the three others for DNA evaluation.

Quantification

Total PGs were extracted from culture media and cell layer at 4°C by incubation with 3 M guanidinium chloride in 0.05 M Tris-HCl (Merck, Darmstadt, Germany) at pH 7.4 in the presence of protease inhibitors: 10 mM Na₂ EDTA (Sigma, St Louis, MO), 5 mM benzamidine (Merck, Darmstadt, Germany), and 0.1 mM 6-aminoheptanoic acid (Prolabo, Fontenay S/Bois, France). Aliquots of the guanidinium extract were then spotted onto a strip of Whatman 3 MM paper and allowed to dry. Each sample was spotted in triplicate. Total [³⁵SO₄]sulphated PG subunits were precipitated on Whatman paper by incubating the strips in a solution containing 5% cetylpyridinium chloride (CPC) in 0.3 M NaCl (Sigma, St Louis, MO) according to Bjelle *et al*,²² as modified by Larsson and Kuettner.²³ The strips were then allowed to dry and each sample was separately cut into pieces. Scintillation liquid was added to each sample, and counted in a Packard tricarb β-spectrometer. DNA was measured in sister flasks by the fluorometric method described by Kapuscinski and Skoczylas.²⁴ Final data are shown as mean (SD) dpm/10 μg DNA from four experiments performed with different animal donors and expressed as a percentage of controls incubated without effectors.

Chromatography

In these experimental conditions 75% newly synthesised radiolabelled PGs are recovered in culture medium. Because it is difficult to separate extra- and intracellular newly synthesised PGs in the remaining 25% of radiolabelled material which is stored on the cell layer, PG degradation was only measured in the culture medium. The elution profile of [³⁵SO₄]sulphated PG secreted by rabbit articular chondrocytes was analysed in IL1β+E2 treated flasks by comparison with flasks treated with IL1β alone, as previously described.²⁵ Radiolabelled PGs were precipitated with 5% CPC in the presence of protease inhibitors: 10 mM Na₂ EDTA (Sigma, St Louis, MO), 5 mM benzamidine (Merck, Darmstadt, Germany), and 0.1 mM 6-aminoheptanoic acid (Prolabo, Fontenay S/Bois, France). After undergoing centrifugation at 3600 g, the pellet was washed with 0.1 M Na₂SO₄ and dissolved with 1.25 M MgCl₂ (Merck, Darmstadt, Germany). The supernatant was centrifuged, precipitated with ethanol (Merck Eurolab, Nogent S/Marne, France), and then left at 4°C overnight. The extracts were lyophilised and dissolved in 0.5 M sodium acetate (pH 6.8) (Calbiochem, La Jolla, CA). Aliquots (500 μl) were applied to a Sepharose 2B (Amersham, Uppsala, Sweden) column (0.5×65 cm) equilibrated in the same buffer with or without excess of non-radioactive carrier. PGs were eluted with 0.5 M

Figure 1 (A) [^{35}S]sulphate incorporation into PGs synthesised and secreted by chondrocytes treated or not with 10 ng/ml IL1 β combined or not with E2 (0.1–10 nmol/l). Articular chondrocytes were cultured until confluency. After 24 hours without FCS, cells were incubated with 1.5 $\mu\text{Ci/ml}$ [^{35}S]sulphate for 20 hours with or without effectors. [^{35}S]sulphated proteoglycans were extracted as described in "Materials and methods". In each experiment six similarly treated flasks were prepared, three being used for the sulphation assay and the other three for DNA measurement. Radioactivity incorporated into [^{35}S]sulphated PG was calculated as mean (SD) dpm/10 μg DNA from four experiments performed with different animal donors and expressed as a percentage of controls incubated without effectors (* $p \leq 0.05$; ** $p \leq 0.01$). (B) Northern blot showing aggrecan mRNA hybridisation signal in chondrocytes treated or not with 10 ng/ml IL1 β combined or not with E2 (0.1–10 nmol/l). Total mRNA was extracted after 20 hours' incubation with or without effectors. The upper part shows the hybridisation signal to the rabbit aggrecan probe and to a human GAPDH probe which indicates the relative amount of RNA loaded in each lane. These data are representative of one of three independent experiments. The lower part shows the densitometric quantification of the autoradiogram expressed as the aggrecan/GAPDH band density ratio. Results are the mean (SD) of three experiments performed with different animal donors.

sodium acetate (pH 6.8) at a flow rate of 2.5 ml/h. Fractions (300 μl) of each eluate were collected and the radioactivity measured by scintillation counting. The total volume (V_t) of the column was determined by the use of free [^{35}S]sulphate. The partition coefficient (K_{av}) of [^{35}S]sulphate in each fraction was calculated as follows:

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

where V_e , V_o , and V_t represent the elution volume for each column fraction, the void, and the total volumes for the column, respectively. For each column, radioactivity eluted at K_{av} 0.3–0.6 (low molecular weight (LMW) PGs) was measured and expressed as a percentage of radioactivity eluted on the column (V_o to V_t). In each experiment two similarly treated flasks were analysed. Results are expressed as mean (SD) of three experiments performed with different animal donors.

Production of rabbit probes by RT-PCR

MMP-1, MMP-3, MMP-13, aggrecan, and tissue inhibitor of metalloproteinase (TIMP) probes were generated by reverse transcriptase-polymerase chain reaction (RT-PCR). To obtain cDNA, a reverse transcriptase (RT) reaction was first performed on 1 μg of total RNA isolated from chondrocytes treated or not by 10 ng/ml IL1 β for 8 hours. The RT reaction was performed using oligo(dT) priming and 200 units of Moloney murine leukaemia virus reverse transcriptase (Promega, France). The resulting cDNA was amplified by PCR using specific primers as described in table 1. Each PCR product was subcloned in pGEMT easy vector (Promega) according to the manufacturer's protocol. DNA sequencing analysis (Perkin Elmer) confirmed the amplification of the appropriate transcript.

RNA isolation and northern blot

After 20 hours' incubation with or without the effectors, total cellular RNA was extracted according to Chomczynski and Sacchi.²⁶ RNA samples (8 μg) were electrophoresed in 1% agarose gel and transferred onto a nylon membrane (GeneScreen, NEN Life Science products). Prehybridisation and hybridisation were performed with the rapid-hyb buffer kit (Amersham, Life Science) according to the manufacturer's protocol.

The rabbit cDNA probes synthesised by RT-PCR amplification were labelled by random priming with ($\alpha^{32}\text{P}$)dCTP (Amersham France) to a mean specific activity of 10^8 – 10^9 cpm/ μg . The hybridised membranes were washed and exposed to Cronex films at -80°C for 16 hours. Quantitative results were performed using scanning densitometry. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as internal control because no variation of chondrocyte GAPDH mRNA level was observed after IL1 β or E2 treatment. Results are the mean (SD) of three or four experiments performed with different animal donors, as indicated in the legends of the figures.

Zymography

Zymographic analysis of gelatinase activities was performed using cell culture supernatants (10 μl). The chondrocyte-conditioned medium, mixed with 4 $\times$ sample buffer (0.25M Tris base 2.5% sodium dodecyl sulphate (SDS), 12.5% glycerol, 0.1% bromophenol blue) was subjected to electrophoresis at 4 $^\circ\text{C}$ on a 0.1% SDS polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gels were incubated in zymogram renaturing buffer (Invitrogen) for 30 minutes, then in zymogram developing buffer (Invitrogen) for 20 hours at 37 $^\circ\text{C}$. Gels were stained with 0.5% Coomassie blue, 10% acetic acid, 30% methanol buffer. A molecular marker was electrophoresed. The levels of the 72 kDa and

Figure 2 (A) Sephadex 2B elution profile of [^{35}S]sulphated proteoglycans synthesised and secreted by chondrocytes treated or not with IL1 β combined or not with E2 (0.1–10 nmol/l). Two similarly treated flasks were prepared in each experiment. Confluent chondrocytes were incubated with [^{35}S]sulphate for 20 hours with or without effectors as described in fig 1. [^{35}S]sulphated PGs were extracted in associative conditions with protease inhibitors as described in “Materials and methods”. Aliquots were applied to a column of Sephadex 2B. Eluates were collected and the radioactivity measured by scintillation counting. V_0 is the void volume and V_t is the total volume of the column. In each experimental condition, LMW radioactive material (hatched grey) eluted between Kav 0.3 and 0.6, is expressed as a percentage of total radioactivity eluted on the column between V_0 and V_t (plain grey). The yield of the column is >90%. Data are representative of a series of flasks examined in one of three independent experiments. (B). For each column, radioactivity eluted at Kav 0.3–0.6 (LMW PG) was measured and expressed as a percentage of the total radioactivity eluted on the column (V_0 to V_t). In each experiment two similarly treated flasks were analysed. Results are expressed as the mean (SD) of three experiments performed with different animal donors (* $p \leq 0.05$).

Figure 3 Northern blot showing the effects of E2 on IL1 β -induced MMP-1, -3, and -13 mRNA hybridisation signals. Confluent chondrocytes were incubated for 20 hours with or without effectors as described above. After 20 hours, total RNA was extracted and analysed as in fig 1B. The upper part shows the hybridisation signal to the rabbit MMP-1, -3, -13 probes and to the human GAPDH probe. Data are representative of one of four independent experiments. The lower part shows the densitometric quantification of the autoradiogram expressed as the MMP/GAPDH band density ratio. Data are the mean (SD) from four independent experiments (* $p \leq 0.05$, ** $p < 0.01$).

53/58 kDa gelatinolytic activity were quantified by video-densitometry. Results are the mean (SD) of four experiments performed with different animal donors.

DNA construction

MMP-1 induction by IL1 β in rabbit fibroblasts requires interaction between activated protein-1 (AP-1) site (-77 bp) and NF κ B-like elements (-3029 bp).²⁸ We thus prepared an MMP-1 promoter construct containing 3.45 kb of the 5' flanking region linked to the luciferase reported gene (pMMP-1-Luc). Rabbit genomic DNA was extracted from chondrocytes with Trizol reagent kit (Gibco BRL, USA) according to the manufacturer's protocol. The 3.45 kb MMP-1 promoter luciferase vector was constructed by using pGL3 basic vector (Promega). The 3.45 kb fragment was amplified by PCR using the following primers: upper 5'-CGGGGTA-CCGACCAAGGACTCAAGA-3' and lower 5'-CCGCTCGAG-TGTCTTCTCTCAGT-3' (Genbank M17820); so the amplified product could be directionally inserted between the *KpnI* and *XhoI* sites of pGL3 vector as described by Vincenti *et al.*²⁸ The orientation and sequence fidelity of the cloned fragments were checked by a 373 DNA sequencer (Perkin Elmer).

Transient transfection and luciferase assay

Rabbit chondrocytes were cultured in Ham's F12 containing 10% FCS as described above, trypsinised, and plated in six

well plates. Cells were then transfected at 70% confluence by a calcium phosphate precipitation method. Briefly, cells were incubated for 4 hours with the precipitate obtained with 0.02 M CaCl₂ in a HEPES buffer pH 7.0 and 1.5 μ g DNA/well—that is, 1 μ g of the MMP-1 promoter-luciferase construct and 0.5 μ g of pRSV β gal, a β -galactosidase expression vector. The medium was replaced with serum-free DMEM to which E2 alone or combined with IL1 β were added. Chondrocytes were further incubated for an additive period of 20 hours. Cells were then lysed in 400 μ l lysis buffer (Promega). Each sample (20 μ l) of cell extracts was used in triplicate for determination of protein content, luciferase, and β -galactosidase activities. The β -galactosidase activity was evaluated with β -galactosidase enzyme assay system (Promega). The luciferase activity was measured on a Lumat LB 9507 luminometer (Berthold). Data were expressed as luciferase activity normalised to the β -galactosidase activity. Three similarly treated wells were prepared for each experiment. Results are the mean (SD) of three experiments performed with different animal donors.

Statistical analysis

Values are expressed as the mean (SD). Statistics were performed by analysis of variance. Multiple comparisons were performed with the Tukey test. Differences between groups were considered significant when $p \leq 0.05$.

Figure 4 (A) Gelatinolytic activities and (B) TIMP-1 mRNA levels in chondrocytes treated or not with 10 ng/ml IL1β combined or not with E2 (0.1–10 nmol/l). Confluent chondrocytes were incubated for 20 hours with or without effectors as described in fig 1. Culture medium was collected and subjected to gelatin zymography as described in “Materials and methods”. Total RNA was extracted from the corresponding cultured flasks and analysed as in fig 1B for TIMP-1 hybridisation signal. (A) Upper part: the gelatinolytic bands are representative of a series of flasks from one of four independent experiments. Lower part: the level of gelatinolytic bands was determined by scanning video-densitometry. Because the 72 kDa band densitometry was not modified by IL1β or E2, this band was used as internal control. The densitometric quantification of the IL1β-induced 53–58 kDa band was thus expressed as the 53–58 kDa/72 kDa band ratio. Data are the mean (SD) of results obtained from four independent experiments performed with different animals. (B) Northern blot analysis of TIMP-1 mRNA hybridisation signal. The upper part shows the hybridisation signal to the rabbit TIMP-1 probe and to a human GAPDH probe. Data are representative of a series of flasks from one of four independent experiments. The lower part shows the densitometric quantification of the autoradiogram expressed as mean (SD) of TIMP-1/GAPDH band density ratio measured in four independent experiments.

RESULTS

17β-Oestradiol modulates IL1β-induced proteoglycan degradation but not aggrecan mRNA level in cultured chondrocytes

Radiolabelled PGs newly synthesised by rabbit articular chondrocytes were extracted from cellular pool and corre-

Figure 5 MMP-1 gene promoter activity. The upper part represents the design of the 3.45 kb of the 5' flanking region linked to the luciferase reported gene used in DNA transfection experiments. Chondrocytes were transiently cotransfected with MMP-1 gene promoter and with a β-galactosidase expression vector and further treated with each effector for 20 hours. Each point was measured in triplicate. The lower part shows the relative luciferase activity calculated as fold induction in treated cells with respect to the basal activity measured in untreated cells. Values for the luciferase activity were normalised to the β-galactosidase activity. Results are the mean (SD) of three independent experiments.

sponding culture medium. Figure 1A shows that in the presence of IL1β, [³⁵SO₄]sulphated PG decreases by 36% from 6.35 (0.56) × 10⁴ dpm/10 μg DNA in control conditions down to 4.12 (0.37) × 10⁴ dpm/10 μg DNA (p<0.05, n=4). Addition of E2 in combination with IL1β modulates the amount of [³⁵SO₄]sulphated PG in a dose dependent manner. When added with IL1β, E2 reproducibly stimulates radiolabelled material by 130% (9.51 (0.45) dpm/10 μg DNA, p<0.01) at 0.1 nmol/l and by 32% (5.43 (0.17) dpm/10 μg DNA, p=0.05) at 1 nmol/l. In contrast, when E2 is added at high concentration (10 nmol/l), [³⁵SO₄]sulphated PGs are decreased by 53% below values observed with IL1β alone (1.92 (0.24) dpm/10 μg DNA, p<0.01), thus enhancing the effect of IL1β.

In addition, exposure of the cells to IL1β resulted in a 30 (7)% decrease (p<0.05) of aggrecan mRNA level as compared with control cells referred to as 100% (fig 1B). Addition of E2 did not modify the IL1β-induced decrease of mRNA aggrecan at any concentration used.

17β-Oestradiol modulates IL1β-induced low molecular weight PGs

The respective proportions of high molecular weight (HMW) PGs, excluded from the column, and LMW PGs, recovered at Kav 0.3–0.6, were then studied. In control conditions (serum-free DMEM) more than 85 (5)% of the ³⁵SO₄ labelled PGs applied on the column were eluted at Kav=0.1–0.3 as HMW aggregated complexes, with less than 10 (4)% eluted at Kav=0.3–0.6 as LMW PGs (figs 2A and 2B). In IL1β treated cells a significantly (p<0.05) increased amount of LMW radioactive PG (26 (6)% of total radioactivity) was seen as compared with control conditions. Addition of 0.1 or 1 nM E2 did not significantly change the amount of LMW

PGs, which represent respectively 17 (4)% and 29 (6)% of the total radioactivity. In contrast, 10 nM E2 significantly ($p < 0.05$) increased the amount of LMW PGs to 39 (3)% of total radioactivity, thus enhancing the degradative effect of IL1 β .

17 β -Oestradiol modulates IL1 β -induced MMP-1, -3, and -13 mRNA expression

Figure 3, upper part, indicates that mRNA expression of MMP-1, -3, and -13 is strongly expressed when cells are treated with IL1 β , whereas no detectable signal is seen in control conditions (serum-free DMEM) or in the presence of E2 (10 nmol/l) alone. Simultaneous addition of IL1 β and E2 to the cultured cells resulted in a biphasic dose-dependent variation of MMP mRNA. With 0.1 nM E2, the level of each MMP transcript is reduced by 30 (3)% ($p = 0.05$) as compared with the mRNA content seen with IL1 β alone. In contrast, addition of higher doses of E2 enhances the effect of IL1 β , thus resulting in a significantly increased MMP-1, -3, and -13 signals up to 41 (5)% ($p < 0.05$) with 1 nM E2 and to 112 (4)% ($p < 0.01$) with 10 nM E2.

17 β -Oestradiol modulates IL1 β -induced gelatinolytic activities

In control conditions chondrocytes secreted one main gelatinolytic activity at an apparent molecular weight of 72 kDa (MMP-2) (fig 4A, upper part). Exposure of the cells to IL1 β resulted in the expression of an additive gelatinolytic activity at an apparent molecular weight of 53–58 kDa, presumably corresponding to MMP-1. Because the 72 kDa band was not modified by IL1 β or E2 treatment, quantification analysis of the 53–58 kDa bands was related to the 72 kDa band. Figure 4A, lower part, shows that the intensity of the 53–58 kDa band varied with E2 concentration in a dose dependent manner, similar to that seen with expression of MMP mRNA. In the presence of low concentrations of E2 (0.1 nmol/l) the 53–58 kDa gelatinolytic activity was reduced by 38 (4)% ($p < 0.05$) in comparison with that seen in the presence of IL1 β alone. In contrast, treatment of the cells with 1 nM or 10 nM E2 significantly increased the IL1 β -induced band by 48 (5)% ($p < 0.05$) and by 105 (4)% ($p < 0.01$), respectively.

Because the level of the 53–58 kDa gelatinolytic activity depends on the balance between MMP and its inhibitors, we then determined whether E2 could modulate the level of TIMP-1 mRNA. As shown in fig 4B, addition of E2 does not significantly modify the TIMP-1 mRNA level.

Effect of 17 β -oestradiol on MMP-1 promoter activity

Chondrocytes were transiently transfected with pMMP-1-Luc and further incubated for 16 hours in serum-free DMEM containing E2 at concentrations ranging from 0.1 to 10 nmol/l, alone or in combination with IL1 β (10 ng/ml). Addition of IL1 β increased luciferase activity sixfold in comparison with cells incubated in serum-free DMEM (fig 5). Simultaneous addition of IL1 β and E2 (0.1–10 nmol/l) did not modify IL1 β -induced luciferase activity. Moreover, addition of E2 alone was unable to induce any luciferase activity in transfected cells, whatever the concentration used.

DISCUSSION

Our data show for the first time that E2, added in combination with IL1 β to cartilage cells in vitro, modulates IL1 β -induced PG degradation, MMP-1, -3, and -13 mRNA expression, and the 53–58 kDa gelatinolytic activity; the modulation is inversely related to the E2 concentration used. At a low concentration of E2 (0.1 nmol/l) IL1 β -induced

effects are inhibited, whereas they are enhanced at high concentration (10 nmol/l).

The biphasic effect of E2 is seen in the IL1 β -induced reduction in the amount of total sulphated PGs evaluated by CPC precipitation. Such an E2 effect is not seen on aggrecan mRNA expression, suggesting that E2 probably does not affect the synthesis of the core protein of the aggrecan. But the present data do not allow us to definitively eliminate the possibility that E2 modulates the synthesis of glycosaminoglycans or other types of sulphated PGs such as decorin, biglycan, fibromodulin, lumican, etc.

On the other hand, the biphasic effect of E2 on PGs may result from an interaction of E2 with the process of PG degradation induced by IL1 β . Our data clearly indicate that high concentrations of E2 potentiate the effect on IL1 β -induced production of LMW PGs as well as the expression of MMPs involved in PG degradation, at both mRNA and protein levels. Low concentrations of E2 (≤ 1 nmol/l) have opposite effects.

When secreted by chondrocytes, neosynthesised sulphated proteoglycans form large molecular size aggregates composed of non-covalent association between hyaluronate, link protein, and the globular G1 domain of aggrecan, resulting in supramolecular aggregates.²⁹ Chondrocytes submitted to cytokines such as IL1 β , express MMPs involved in the dissociation of the hyaluronate binding G1 domain of aggrecan that results in disaggregation of PGs, which can be visualised and quantified on the elution profile of Sepharose 2B column chromatography.^{29–30} E2 added with IL1 β at 10 nmol/l significantly augments the disaggregating effect of IL1 β by increasing the extent of LMW PGs up to 50% above values seen with IL1 β alone. No convincing opposite effect of a low concentration of E2 is found for this measure. The 10% decreased amount of LMW PGs seen with 0.1 nM E2 is not significant. Increasing the number of experiments would have probably improved these data. However, a clear demonstration of the biphasic effect is found at the next step performed to study whether MMPs involved in PG cleavage are modulated by high and low concentrations of E2.

Three main MMPs—namely, MMP-1, -3, and -13, are involved in the IL1 β -induced degradation of cartilage matrix proteins³¹ and have been shown to cleave aggrecan within the interglobular domain at the similar Asn341-Phe342 peptide bond.^{32–33} One can observe that E2 acts with a significant biphasic and dose dependent effect on the expression of MMP-1, -3, and -13 transcripts, as well as on the 53–58 kDa gelatinolytic activity (MMP-1). The IL1 β -induced amount of MMP mRNAs and MMP-1 gelatinolytic activity are twofold lower with 0.1 nM E2 and 2.5-fold higher with 10 nM E2. E2 does not seem to act by modulating MMP inhibitors because E2 does not affect the level of TIMP-1 mRNA. These results are in accordance with those of Kapila *et al.*,³⁴ who reported a dose dependent modulation of 53–58 kDa gelatinolytic activity by E2 in temporomandibular cartilage cells in vitro, which was not associated with significant modulation of TIMP-1. Liao *et al.* also reported that E2 caused a dose dependent decrease in MMP-1 production, with no influence on TIMP-1 expression, in osteoblast-like cells in culture.²⁰ Interestingly, because we show that a high concentration of E2 (10 nmol/l) maximises the expression of MMP mRNAs induced by IL1 β , such a potentiality of induced MMP expression has also been shown in other cell types such as UMR 106-01 osteosarcoma cells treated with parathyroid hormone³⁵ and in rat fibrocartilaginous cells treated with relaxin.³⁴

In the classical signalling pathway, the oestrogen receptor binds to oestrogen response elements at the promoter of target genes and stimulates transcription either directly or

indirectly through cofactors.³⁶ An oestrogen-receptor complex can also activate genes by interacting with an AP-1 responsive element.³⁷

We focused on the MMP-1 gene and suggested that E2 could modulate MMP-1 expression through IL1 β transcription factors. It is likely that IL1 β action on MMPs occurs via AP-1 and/or NF κ B transcription factors.²⁸ Cells were transiently transfected with a fragment of 3.45 kb MMP-1 promoter containing AP-1 and NF κ B, linked to the luciferase reporter gene (pMMP-1-Luc). No effect of E2 alone or combined with IL1 β is observed on the MMP-1 promoter activity whatever E2 concentrations are used. In addition, E2 probably does not interfere with the dynamic of the transcriptional machinery of MMP-1 because preincubation of the cells with E2 for 1, 3, or 6 hours does not modify the results (data not shown). Further studies are needed to investigate whether E2 acts at the post-transcriptional level on MMP genes as suggested by Partridge *et al* in osteosarcoma cells.³⁵ Such a hypothesis has been well demonstrated for the transcripts of *Xenopus* and chicken genes, such as vitellogenin,³⁸ albumin,³⁹ and ovalbumin,⁴⁰ which are regulated through stabilisation by E2, probably through RNA binding proteins involved in the regulation of RNA processing.

In the present experimental conditions, E2 modulates IL1 β -induced effects on PG degradation and de novo expression of MMPs in isolated cultured chondrocytes. Even though the present in vitro data have to be confirmed in vivo, one can suggest that E2 may modulate cartilage degradation in IL1 β -induced inflammatory joints in vivo. An increased level of MMPs, together with a significant expression of cytokines such as IL1 β , have been seen in synovial fluids from humans with OA.³¹ However, there are no data available concerning the synovial fluid concentration of E2 in osteoarthritic women. Considering the dual effect of E2 on IL1 β -induced cartilage degradation, it might be of interest to study the level of oestrogen concentration in the synovial fluid of osteoarthritic women receiving oestrogen replacement therapy. In the case of E2 physiological levels, it is likely that oestrogen replacement therapy may have beneficial effects by decreasing the cartilage degradation process. Conversely, one must keep in mind that administration of high oestrogen concentration might have opposite effects and be deleterious to cartilage by increasing IL1 β -induced PG degradation. The clinical significance of this latter finding remains to be further investigated. In conclusion, our in vitro findings shed light on the importance of oestrogen concentration that may have contradictory effects on IL1 β stimulated chondrocytes.

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REFERENCES

- 1 Felson DT, Zhang Y. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. *Arthritis Rheum* 1998;**41**:343–55.
- 2 Wluka AE, Cicuttini FM, Spector TD. Menopause, oestrogens and arthritis. *Maturitas* 2000;**30**:183–99.
- 3 Mori JS, Billington CJ. Articular cartilage and changes in arthritis: matrix degradation. *Arthritis Res* 2001;**3**:337–41.

- 4 Mengshol JA, Mix KS, Brinckerhoff CE. Matrix metalloproteinases as therapeutic targets in arthritic diseases: bull's-eye or missing the mark? *Arthritis Rheum* 2002;**46**:13–20.
- 5 Tello LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum* 2001;**44**:585–94.
- 6 Little CB, Hughes CE, Curtis CL, Janusz MJ, Bohne R, Wang-Weigand S, *et al*. Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation. *Matrix Biol* 2002;**21**:271–88.
- 7 Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms involved in cartilage proteoglycan catabolism. *Matrix Biol* 2000;**19**:333–44.
- 8 Ushiyama T, Ueyama H, Inoue K, Ohkubo I, Hukuda S. Expression of genes for estrogen receptors alpha and beta in human articular chondrocytes. *Osteoarthritis Cartilage* 1999;**7**:560–6.
- 9 Dayani N, Corvol MT, Robel P, Eychemme B, Monchamont B, Tsagris L, *et al*. Estrogen receptors in cultured rabbit articular chondrocytes: influence of age. *Steroid Biochem* 1988;**31**:351–6.
- 10 Richmond RS, Carlson CS, Register TC, Shanker G, Loeser RF. Functional estrogen receptors in adult articular cartilage: estrogen replacement therapy increases chondrocyte synthesis of proteoglycans and insulin-like growth factor binding protein 2. *Arthritis Rheum* 2000;**43**:2081–90.
- 11 Turner AS, Athanasios KA, Zhu CF, Alvis MR, Bryant HU. Biochemical effects of estrogen on articular cartilage in ovariectomized sheep. *Osteoarthritis Cartilage* 1997;**5**:63–69.
- 12 Ham KD, Loeser RF, Lindgren BR, Carlson CS. Effects of long-term estrogen replacement therapy on osteoarthritis severity in cynomolgus monkeys. *Arthritis Rheum* 2002;**46**:1956–64.
- 13 Femihough JK, Richmond RS, Carlson CS, Cherpes T, Holly JM, Loeser RF. Estrogen replacement therapy modulation of the insulin-like growth factor system in monkey knee joints. *Arthritis Rheum* 1999;**42**:2103–11.
- 14 Corvol MT, Carrascosa A, Tsagris L, Blanchard O, Rappaport R. Evidence for a direct in vitro action of sex steroids on rabbit cartilage cells during skeletal growth: influence of age and sex. *Endocrinology* 1987;**120**:1422–9.
- 15 Blanchard O, Tsagris L, Rappaport R, Duval-Beaupere G, Corvol MT. Age-dependent responsiveness of rabbit and human cartilage cells to sex steroids in vitro. *J Steroid Biol* 1991;**40**:711–16.
- 16 Ng MC, Harper RP, Le CT, Wong BS. Effects of estrogen on the condylar cartilage of the rat mandible in organ culture. *J Oral Maxillofac Surg* 1999;**57**:818–23.
- 17 Rajabi M, Solomon S, Poole AR. Hormonal regulation of interstitial collagenase in the uterine cervix of the pregnant guinea pig. *Endocrinology* 1991;**128**:863–71.
- 18 Potier M, Elliot SJ, Tack I, Lenz O, Striker GE, Striker LJ, *et al*. Expression and regulation of estrogen receptors in mesangial cells: influence on matrix metalloproteinase-9. *J Am Soc Nephrol* 2001;**12**:241–51.
- 19 Guccione M, Silbiger S, Lei J, Neugarten J. Estradiol upregulates mesangial cell MMP-2 activity via the transcription factor AP-2. *Am J Physiol Renal Physiol* 2002;**282**:F164–9.
- 20 Liao EY, Luo XH. Effects of 17 β -estradiol on the expression of matrix metalloproteinase-1, -2 and tissue inhibitor of metalloproteinase-1 in human osteoblast-like cell cultures. *Endocrine* 2001;**15**:291–5.
- 21 Corvol MT, Dumontier MF, Rappaport R. Culture of chondrocytes from the proliferative zone of epiphyseal growth plate cartilage from prepubertal rabbits. *Biomedicine* 1975;**23**:103–7.
- 22 Bijelle A, Gardell S, Heinegard D. Proteoglycans of articular cartilage from bovine lower femoral epiphysis. Extraction and characterisation of proteoglycans from two sites within the same joint. *Connect Tissue Res* 1974;**2**:111–16.
- 23 Larsson SE, Kuettner KE. Microchemical studies of acid glycosaminoglycans from isolated chondrocytes in suspension. *Calcif Tissue Res* 1974;**14**:49–58.
- 24 Kapuscinski J, Skoczylas B. Simple and rapid fluorimetric method for DNA microassay. *Anal Biochem* 1977;**83**:252–7.
- 25 Rannou F, Corvol MT, Hudry C, Anract P, Dumontier MF, Tsagris L, *et al*. Sensitivity of anulus fibrosus cells to interleukin 1 beta. Comparison with articular chondrocytes. *Spine* 2000;**25**:17–23.
- 26 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156–9.
- 27 Hellio Le Graverand MP, Reno C, Hart DA. Influence of pregnancy on gene expression in rabbit articular cartilage. *Osteoarthritis Cartilage* 1998;**6**:341–50.
- 28 Vincenti MP, Coon CI, Brinckerhoff CE. Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1 beta-stimulated synovial fibroblasts. *Arthritis Rheum* 1998;**41**:1987–94.
- 29 Schwartz NB, Pirok EW 3rd, Mensch JR Jr, Domowicz MS. Domain organization, genomic structure, evolution, and regulation of expression of the aggrecan gene family. *Prog Nucleic Acid Res Mol Biol* 1999;**62**:177–225.
- 30 Sajdera SW, Hascall VC. Proteinopolysaccharide complex from bovine nasal cartilage. A comparison of low and high shear extraction procedures. *J Biol Chem* 1969;**244**:77–87.
- 31 Martel-Pelletier J. Pathophysiology of osteoarthritis. *Osteoarthritis Cartilage* 1999;**7**:371–3.
- 32 Deng SJ, Bickett DM, Mitchell JL, Lambert MH, Blackburn RK, Carter HL 3rd, *et al*. Substrate specificity of human collagenase 3 assessed using a phage-displayed peptide library. *J Biol Chem* 2000;**275**:31422–7.

- 33 **Fosang AJ**, Last K, Knauper V, Murphy G, Neame PJ. Degradation of cartilage aggrecan by collagenase-3 (MMP-13). *FEBS Lett* 1996;**380**:17–20.
- 34 **Kapila S**, Xie Y. Targeted induction of collagenase and stromelysin by relaxin in unprimed and beta-estradiol-primed diarthrodial joint fibrocartilaginous cells but not in synoviocytes. *Lab Invest* 1998;**78**:925–3.
- 35 **Partridge NC**, Fiacco GJ, Walling HW, Barmina OY, Jeffrey JJ, Ruh MF. Effects of dioxin and estrogen on collagenase-3 in UMR 106-01 osteosarcoma cells. *Arch Biochem Biophys* 2000;**382**:182–8.
- 36 **McKenna NJ**, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocrinol Rev* 1999;**20**:321–44.
- 37 **Hall JM**, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999;**140**:5566–78.
- 38 **Dodson RE**, Shapiro DJ. Vigilin, a ubiquitous protein with 14 K homology domains, is the estrogen-inducible vitellogenin mRNA 3'-untranslated region-binding protein. *J Biol Chem* 1997;**272**:12249–52.
- 39 **Pastori RL**, Moskaitis JE, Buzek SW, Schoenberg DR. Coordinate estrogen-regulated instability of serum protein-coding messenger RNAs in *Xenopus laevis*. *Mol Endocrinol* 1991;**5**:461–8.
- 40 **Arao Y**, Kikuchi A, Ikeda K, Nomoto S, Horiguchi H, Kayama F. A+U-rich-element RNA-binding factor 1/heterogeneous nuclear ribonucleoprotein D gene expression is regulated by oestrogen in the rat uterus. *Biochem J* 2002;**361**:125–32.

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