Effects of Estrogen on Chondrocyte Proliferation and Collagen Synthesis in Skeletally Mature Articular Cartilage

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Purpose: Estrogen has been shown to have a modulating effect on cartilage thickness. This investigation was performed to determine the effects of estrogen supplementation on cartilage thickness, cellular proliferation, and type II and X collagen production in skeletally mature rat cartilage, both in an organ culture and cell culture system.

Materials and Methods: Mandibular condyles were harvested from 8-week-old female Sprague Dawley rats and placed into tissue culture plates containing culture media with or without 17β-estradiol supplementation. Organ cultures were labeled with 5-bromo-2′-deoxyuridine on culture day 2 or 4 to determine the effects of estrogen supplementation on the cellular mitotic index. Histomorphometric analysis of the organ culture sections was used to determine the thickness (μm) of the various cartilage zones, as well as the total cartilage thickness following estrogen exposure. Type X collagen was immunohistochemically identified in the ECM of hypertrophic chondrocytes using a rabbit anti-rat collagen type X antibody raised against the NCl domain. The reaction was visualized with an avidin-biotin peroxidase detection system (Vector Laboratories, Burlingame, CA). In a separate experiment, articulating cartilage chondrocytes were harvested by collagenase digestion and cultured at 5×10^5 cells per 35 mm tissue culture plate. Second subculture chondrocytes were divided into 2 groups: controls and [10^{-8} M] 17β-estradiol (E_2-10^{-8} M) and grown to confluence. The cell cultures were used to establish growth curves for each group using cell counts at 2-day intervals.

Results: In the organ culture experiment, 17β-estradiol–treated condyles had a significant decrease in total cartilage thickness after 4 days in culture ($P < .05$). Estrogen supplementation resulted in a significant reduction in the mitotic index as early as culture day 2 ($P < .05$). Type X collagen deposition into the extracellular matrix was visibly increased in the hypertrophic chondrocyte zone for the estrogen-supplemented group on experimental days 2 and 4 compared with the control group. In the cell culture system, 17β-estradiol [10^{-8} M] decreased chondrocyte proliferation during logarithmic growth ($P < .05$) and at confluence ($P < .05$).

Conclusion: These data show that estrogen decreased cartilage thickness by inhibition of chondrocyte proliferation and increased chondrocyte maturation. These observed effects showed the potential role of estrogen in the modulation of skeletally mature cartilage.
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Estrogen has been well established as a major contributor to the regulation of bone growth and development. The effects of estrogen are mediated directly on the cells affecting chondrocyte proliferation, differentiation, and extracellular matrix (ECM) synthesis,[1-4] and indirectly by other hormones and local factors secreted by cells in response to estrogen stimulation.[5,6] It has been traditionally accepted that estrogens mediate their effects through classic steroid–hormone receptor mechanisms. Following estrogen binding to its specific receptor (either estrogen receptor-α [ERα] or estrogen receptor-β [ERβ]),[7-9] the hormone receptor complex translocates to the nucleus where it affects cellular function.[10] Recently, there is increasing evidence that the mechanisms of estrogen

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receptor signaling may involve a nongenomic pathway. The nongenomic actions of 17β-estradiol that have been reported include the mobilization of intracellular calcium,[11] and the stimulation of adenylate cyclase activity and cAMP production.[12,13] Although these experimental findings have been studied in a variety of cell lines, they have not been shown in chondrocytes. In growth cartilage, such as the epiphyseal growth plate or the costochondral growth zone, 17β-estradiol has been shown to inhibit chondrocyte proliferation and stimulate RNA synthesis, sulfate incorporation (matrix synthesis), collagen production, alkaline phosphatase (differentiation), and creatine kinase–specific activities.[3,4,14] These findings are highly dependent on numerous experimental factors and thus remain controversial.

Investigations addressing the effects of ovariectomy on articular cartilage have shown an increased articular soft tissue thickness with a significantly decreased bone volume 1 week after ovariectomy in growing rats.[15] In our previous study,[16] we showed the effects of 17β-estradiol on decreased proteoglycan content of mandibular condylar cartilage in organ culture. Others have studied the effects of 17β-estradiol supplementation on articular cartilage compressive stiffness following oophorectomy.[17] They found that cartilage stiffness and thickness was significantly higher than that for controls. These results, together with previous findings, provided additional insight into the inhibitory effects of 17β-estradiol on the composition of articular cartilage ECM in young skeletally mature animals. Therefore, a change in estrogen concentrations during late maturation of an organism may have a significant effect on cartilage ECM, thereby influencing the load tolerance of articular cartilage during activity and its potential for degeneration.[17]

The importance of understanding the effects of estrogen supplementation on skeletally mature tissues is evident. The effects of 17β-estradiol on articular cartilage chondrocytes from skeletally mature animals, established by fused epiphyseal growth plates, have not yet been clearly addressed in the literature. The purpose of this research, therefore, was to evaluate the effects of 17β-estradiol on the modulation of articular cartilage in the skeletally mature animal. Specifically, the effects of 17β-estradiol on the secondary cartilage of the mandibular condyle were studied with respect to cartilage thickness, chondrocyte proliferation, and ECM production of type X collagen.

Materials and Methods

ORGAN CULTURE

Female Sprague Dawley (SD) rats (Harlan Laboratories, Houston, TX) weighing 175 to 200 g, synchronized in their estrous cycle, were killed after carbon dioxide inhalation. The mandibular condyles were dissected, rinsed in phosphate-buffered saline (PBS), and randomly transferred to labeled 35 mm culture plates containing Phenol Red-free RPMI media supplemented with 10% fetal calf serum, 50 μg/mL L-ascorbic acid, and 1% penicillin/streptomycin. The condylar organ cultures were randomly divided into controls and 17β-estradiol (10^{-8} M)–supplemented groups. Cultures were maintained at 37°C and 5% CO_2. The media was changed daily. It should be noted that control groups would include 10^{-11} M estrogen as a component of the 10% fetal calf serum. Also, the use of tissue culture–treated plates did not cause attachment of the condyles to the dish because gentle agitation was easily achieved during daily media changes.

On experimental days 2 and 4, condyles were removed from the tissue culture–treated plates, rinsed in PBS, and fixed in 4% paraformaldehyde in PBS for 12 hours. The condyles were washed in PBS and transferred to sterile tubes containing 0.5 M EDTA for demineralization over a period of 5 days at 4°C. Tissues were dehydrated and embedded in paraffin. Sagittal paraffin sections were obtained at the midpoint of the condylar head for each condyle and 3 condyles from each group were collected at 2 and 4 days and prepared for histomorphometric and immunohistochemical analysis.

HISTOMORPHOMETRIC ANALYSIS

Condylar sections from the 3 animals in each experimental group were stained with hematoxylin-eosin and photographic slides were made using the Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany) at ×20 magnification. Sections used for histomorphometric analysis were obtained from the thickest mid-sagittal plane of the condylar heads The photographic slides were then scanned using a DuoScan 1200 scanner (AGFA, Mortsel, Belgium) and analyzed with the Optimas software (Bioscan, Edwards, WA). Briefly, a standardized vertical framework of 3 lines was created at a distance of 175 μm apart and positioned over the midpoint region of each image (Fig 1). Next, the various cartilaginous zones, as described by Carlson et al,[18] were manually traced as horizontal lines within this framework. Linear measurements were made of the constituent zone heights, as well as the total cartilage thickness, and measurements were recorded for statistical analysis. Six measurements for each zone were used to calculate an average measure of cartilage thickness differences between the experimental and control groups. The mean height (± SEM) of each zone for the 3 animals in each group was calculated and recorded for statistical comparison.

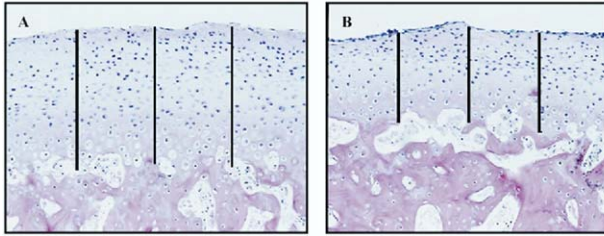

FIGURE 1. Histologic sections (original magnification ×20) of the (*A*) control and (*B*) estrogen-supplemented mandibular condyles from organ culture are represented above. The sections were stained with hematoxylin-eosin, photographic slides were made, and measurements of cartilage thickness (μm) were recorded. A standardized overlay of 3 vertical lines, approximately 175 μm apart, was used for all histologic specimens. Histomorphometric measurements of zonal and total condylar cartilage thickness were made using the Optimas software (Bioscan).

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IMMUNOHISTOCHEMISTRY - BrdU ASSAY

Before collection of organ culture samples on experimental days 2 and 4, respectively, the cultures were pulsed with bromodeoxyuridine (BrdU; Boehringer Manheim Kit No. 85329920-10 exp. 12/00; Indianapolis, IN) for a period of 1 hour. Briefly, the culture media was aspirated from each organ culture dish and replaced with fresh media containing 10 μm BrdU labeling reagent. The organ cultures were washed in 1× washing buffer (BrdU) for 15 minutes at 37°C, 5% CO_2. After washing, the cultures were rinsed in PBS and transferred to sterile 15 mL polypropylene tubes containing 4% paraformaldehyde, avoiding contamination, and processed for histologic analysis.

An immunohistochemical assay for the detection of BrdU labeling within the condylar sections was performed. Tissue sections from the midpoint of each condylar head were used for the BrdU detection assay. Rehydrated and deparaffinized sections were washed 3 times with washing buffer (BrdU) and carefully dried. Fifty microliters of anti-BrdU working solution (BrdU) was added to each specimen and the slides were incubated for 30 minutes at 37°C in the humid chamber. Specimens were again washed 3 times with wash buffer and incubated with 50 μL anti-mouse-Ig-Ap working solution (BrdU) for 30 minutes at 37°C in the humid chamber. Washes were repeated and the specimens were covered with 50 μL of freshly prepared color-substrate solution (BrdU) and incubated at 15°C for 30 minutes. The color-substrate solution was removed by washing specimens with a sufficient amount of washing buffer. The specimen slides were dried, mounted with an aqueous mounting media (AquaMont; Lerner Laboratories, Pittsburgh, PA), and evaluated with light microscopy.

Optimus software (Bioscan) was used to determine the percentage of cells labeled with BrdU in each specimen, creating a mitotic index (MI) for each sample. The MI is defined as the percentage of BrdU-labeled nuclei per total cell count within a standardized area for each specimen (Fig 2).

IMMUNOHISTOCHEMISTRY - TYPE X COLLAGEN

A rabbit anti-rat collagen type X antibody raised against the NCl domain of the collagen molecule was used.[19-21] Deparaffinized sections from the midpoint of the condyles were digested with 2.5% bovine testes hyaluronidase (Sigma, St. Louis, MO) in PBS for 1 hour at 37°C. The sections were incubated with collagen type X antiserum (1:200 dilution) in PBS and 0.1% TritonX-100 overnight at 4°C. The sections were then incubated with goat anti-rabbit antiserum for 1 hour at room temperature. Finally, an avidin-biotin peroxidase detection system (Vector Laboratories, Burlingame, CA) was used to visualize the bound antibody and photographic slides (Ektacrome-100 film; Kodak, Rochester, NY) were made using the Zeiss Axiophot photomicroscope (Zeiss) at a ×20 magnification.

CELL CULTURE PREPARATION

Articulating cartilage was carefully removed from the condylar heads of estrous synchronized female SD rats at 175 g under a dissecting microscope and digested in 2.5% Trypsin and 20 mg/mL collagenase in

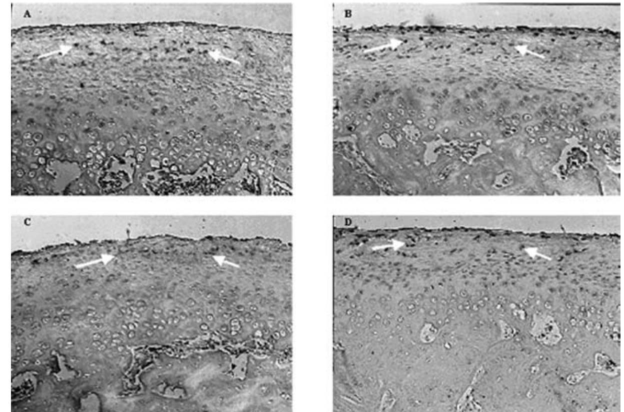

FIGURE 2. Detection of BrdU labeling in histologic section of mandibular condyles from organ culture for the control (*A*, day 2; *B*, day 4) and estrogen-supplemented (*C*, day 2; *D*, day 4) groups. The white arrows in each of the above figures indicate chondrocytes labeled with BrdU. Optimus software (Bioscan) was used to analyze each digital image for positively labeled cells and hence generate a MI for the percentage of BrdU-labeled nuclei per total cell count within a standardized area for each specimen. Actual values of the MI for all specimens are presented in Table 2. Estrogen had an inhibitory effect on the number of BrdU-labeled cells and/or the MI on both experimental day 2 (*C*) and day 4 (*D*) in organ culture, as compared with controls (*A* and *B*).

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PBS for 5 30-minute intervals. The supernatant was collected at each 30-minute interval, centrifuged at 3,500 rpm for 10 minutes, and the cell pellet was resuspended in PBS. Centrifugation and resuspension was repeated twice and the cell pellet was finally resuspended in complete media (Phenol Red-free RPMI, 10% fetal calf serum, 1% penicillin/streptomycin) and plated in 35 mm tissue culture–treated dishes. Cell cultures were confluent within 2 weeks and were subsequently subcultured into 150 mm culture flasks and grown to confluence. All experiments were performed on second passage cell colonies.

CELL PROLIFERATION

Articulating cartilage chondrocytes from second-passage cultures were used to determine the effects of 17β-estradiol on cellular proliferation. Cultures were defined as containing chondrocytes based on morphology, alcian blue stain, and type II collagen production. Cells were trypsinized from the 150 mm tissue culture–treated flasks and collected in 50 mL centrifuge tubes. The cell suspension was centrifuged at 3,000 rpm for 10 minutes and the cell pellet was resuspended and washed twice in PBS. After washing, the cells were resuspended in complete media as described above and a cell count was obtained using a hemocytometer (Hausser Scientific, Horsham, PA). Cells were then plated at an initial concentration of 5×10^5 cells per 35 mm culture dish and divided into 2 experimental groups: controls and 10^{-8} M 17β-estradiol treated ($E_2 - 10^{-8}$ M). Each group contained 6 cell culture plates for every 2-day testing interval, totaling 36 dishes in each group for the 12-day study. At each 2-day interval, media was collected from each of the 6 plates per group and stored at -20°C until further analysis. Also, the cell layer was trypsinized and the cells collected from 6 plates per group, counted using a hemocytometer, and prepared for a DNA assay.

DNA ASSAY

Cell cultures from the control and experimental groups were trypsinized, collected, washed twice in PBS, and centrifuged in sterile glass tubes. The cell precipitates/pellets were solubilized with 1.0 mL of 1% solution of Triton-X and incubated for 3 hours. Eight DNA standards ranging in concentration from 0 to 15 μg/mL were prepared from a stock solution of Salmon Sperm DNA (10 mg/mL). For 1 mL of sample or standards, 175 μL of 70% perchloric acid was added and heated at 74°C for 20 minutes in a water bath. In a separate glass vial, 2 g of diphenylamine was dissolved in 50 mL of glacial acetic acid and 1.0 mL of this solution was added to each of the sample or standard tubes. Then, 500 μL of acetaldehyde was added to 250 mL of distilled water in a sterile vial and 50 μL of this dilution was added to each tube and capped tightly with a stopper. The sample and standard tubes were covered with foil and the color was allowed to develop overnight at room temperature. All of the tubes were centrifuged at 3,000 rpm for 5 minutes and the optical density of the supernatant was read for each tube at 596 nmol/L.

STATISTICAL ANALYSIS

The data presented in this study were analyzed using the SPSS statistical program (Version 10.0; SPSS, Chicago, IL). Data was analyzed using a 1-way analysis of variance (ANOVA) to determine significant differences among the groups. Significance was determined at a level of $P \leq .05$.

Results

EFFECTS OF ESTROGEN ON MANDIBULAR CONDYLAR CARTILAGE IN ORGAN CULTURE

Histomorphometry

When mandibular condyles were isolated from female SD rats and placed in organ culture to evaluate the effects of 17β-estradiol [10^{-8} M] on the condylar cartilage they appeared to undergo histologic changes, which were observed microscopically. All of the condyles remained unattached to the culture plate surface throughout the experiment. Also, no gross structural changes were observed for any of the condyles. All of the specimens in the experimental group were compared histomorphometrically with control group using the Optimus software (Bioscan). Following 2 days in culture, there was a significant reduction in the thickness of the hypertrophic zone for the 17β-estradiol [10^{-8} M]–treated group (79.3 ± 3.8 μm) as compared with controls (108.4 ± 7.4 μm). Interestingly, there was no significant difference in total mandibular condylar cartilage thickness between the experimental and control groups on experimental day 2 (Table 1). After 4 days in culture, the 17β-estradiol [10^{-8} M]–treated group had a significant decrease ($P < .05$) in the total cartilage thickness (Fig 3) (190.8 ± 12.5 μm) compared with controls (361.9 ± 10.7 μm). In these specimens, all of the cartilaginous zones were significantly reduced in thickness in the estrogen-supplemented group (Fig 3).

Mitotic Index (MI)

Immunohistochemical analysis was performed for the detection of BrdU-labeled proliferating chondrocytes for both the control and experimental condyles in organ culture (Fig 2). Scanned images of the BrdU-labeled histologic sections were analyzed using the Optimus software (Bioscan). Cells labeled with BrdU appeared more densely stained in each image and

Table 1. MEAN CARTILAGE THICKNESS (μM) ± SEM

Group	Articular Zone	Proliferative Zone	Maturation Zone	Hypertrophic Zone	Total
Control day 2	33.3 ± 4.0	56.3 ± 7.6	98.1 ± 2.9	108.4 ± 7.4	292.2 ± 18.8
17β-E_2 day 2	40.3 ± 1.9	69.7 ± 2.6	79.5 ± 2.8	79.3 ± 3.8*	264.4 ± 8.7
Control day 4	40.6 ± 2.5	83.0 ± 4.3	113.6 ± 2.9	123.6 ± 5.6	361.9 ± 10.7
17β-E_2 day 4	15.7 ± 1.2*	49.4 ± 3.9*	53.7 ± 5.2*	70.5 ± 3.4*	190.8 ± 12.5*

NOTE. Histologic sections from the mandibular condyles in organ culture were analyzed using the Optimas software (Bioscan, Edwards, WA) for the effects of estrogen supplementation of cartilage thickness. Data represent mean cartilage thickness (μm) ± standard error (SEM) for control and estrogen-supplemented [10^8M] groups (17β-E_2) on experimental days 2 and 4, for the various cartilaginous zones and the total cartilage thickness. Estrogen supplementation resulted in a significant reduction in the thickness of the hypertrophic zone on both experimental day 2 and 4, as compared with controls. The total cartilage thickness was found to be significantly reduced for the estrogen-treated group at the later experimental time period (day 4).

*Significant difference from controls ($P < .05$).

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could therefore be detected by the software. The Optimus program was then able to automatically calculate the approximate percentage of BrdU-labeled cells from total number of cells in each specimen, creating an MI for each sample. The average MI for the control and 17β-estradiol [10^{-8} M]–supplemented group is listed in Table 2, column 4, for both experimental days 2 and 4. Column 4 of Table 2 represents the average of the mitotic indices calculated for each of the 3 specimens in all experimental groups. There was a significant reduction (represented by the asterisk, $P < .05$) in the number of proliferating chondrocytes observed for the 17β-estradiol [10^{-8} M]–supplemented condyles, as compared with controls, as early as 2 days in organ culture. This significant difference persisted for the 17β-estradiol [10^{-8} M]–supplemented group until experimental day 4.

Immunohistochemistry - Type X Collagen

The expression of type X collagen, a marker for hypertrophic (mature) chondrocytes, was investigated by immunohistochemistry (Fig 4). Qualitative observations were made for the difference in type X collagen labeling for both the control and 17β-estradiol [10^{-8} M]–supplemented groups on experimental days 2 and 4. There was a visible increase in the labeling for type X collagen, initially evident on experimental day 2, within the hypertrophic chondrocyte zone for the 17β-estradiol [10^{-8} M]–supplemented condylar sections (Fig 4B). The intensity of labeling for type X collagen was even more distinct in the 17β-estradiol [10^{-8} M]–supplemented sections on experimental day 4 (Fig 4C), as compared with the control condylar sections (Fig 4A). Also apparent at the later experimental time period for the 17β-estradiol [10^{-8} M]–supplemented group (Fig 4C), was an advancing mineralization front with entrapped hypertrophic chondrocytes expressing type X collagen in their immediate ECM environment. The cartilaginous disc was used as a negative control for the type X collagen immunohistochemical assay (data not shown).

EFFECTS OF ESTROGEN ON ARTICULAR CHONDROCYTES

Chondrocyte Proliferation

Using chondrocytes isolated from condylar cartilage, a cell culture system was established to test the effects of estrogen on chondrocyte proliferation. As a result, a growth curve was established for the effects of 17β-estradiol [10^{-8} M] supplementation on the cultured condylar chondrocytes. Figure 5 illustrates the effect of this supplementation on the total number of chondrocytes in cell culture over a 12-day testing period. All cultures were started at an initial concentration of 5×10^5 cells per 35 mm plate. Estrogen had an inhibitory effect on cellular proliferation during the early logarithmic growth phase (day 4), with a significant decrease ($P < .05$) in cell number observed between control ($5.1 \pm 0.4 \times 10^5$ cells/plate) and the estrogen-treated group ($3.5 \pm 0.1 \times 10^5$ cells/plate) (Fig 5).

All groups of chondrocytes reached confluence after approximately 8 days in culture, with an observable ECM surrounding the cells and the appearance of a 3-dimensional layering of the cells. The morphologic characteristics of the control cell culture were observed at various time points along the growth curve. At confluence (≥ day 8), the number of cells in the control group ($12.6 \pm 0.8 \times 10^5$ cells/plate) was significantly greater than in the estrogen-supplemented group ($7.6 \pm 0.3 \times 10^5$ cells/plate) ($P < .05$). Further, this significant decrease in cell number was maintained for the cells in the estrogen treatment group at the later time intervals ($P < .05$).

Discussion

Previous experimental work addressing the effects of estrogen supplementation on chondrocyte prolif-

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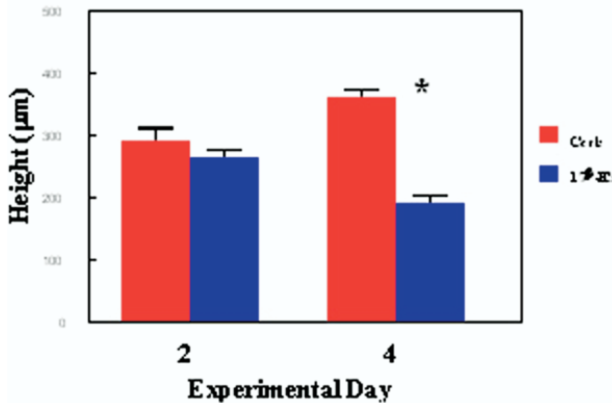

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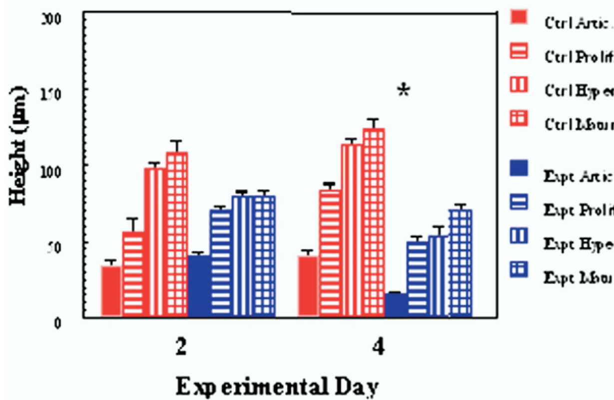

FIGURE 3. Effects of 17β-estradiol [10^{-8} M] supplementation on condylar cartilage thickness of mandibular condyles isolated from female SD rats and maintained in organ culture for 2 and 4 days, respectively. Measurements (μm) of total cartilage thickness (*A*) and of the various cartilaginous zones (*B*). Controls (Ctrl), experimental (Expt), articular zone (Artic), proliferative zone (Prolif), hypertrophic zone (Hyper), and maturation zone (Matur) are represented at the 2 experimental time intervals. Estrogen caused a significant decrease in total cartilage thickness on experimental day 4, while only the hypertrophic zone was significantly reduced in thickness on experimental day 2. *Significant difference from controls ($P < .05$).

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eration and differentiation has primarily been performed on chondrocytes from primary cartilages in the developing organism.[2-4,22] These types of studies have assisted investigators in better understanding various growth-related disorders resulting from hormonal influence.[1] More recently, Ng et al[16] documented the inhibitory effects of estrogen supplementation on proteoglycan content in the ECM of the mandibular condylar cartilage of the skeletally mature rat. Changes in the cartilage ECM have also been shown to influence load tolerance of articular cartilages, thereby increasing its potential for degeneration.[17] These earlier findings led to the need for further investigation on the cellular and environmental events resulting from estrogen supplementation of skeletally mature articulating cartilages.

Therefore, this investigation was designed to evaluate the effects of estrogen supplementation on cellular proliferation, ECM production of type X collagen, and changes in cartilage morphology for the articulating cartilage in the skeletally mature organism. By using both an organ culture and cell culture system, we were able to show similar and complementary inhibitory effects of 17β-estradiol on articulating cartilage chondrocytes, thereby establishing 2 experimentally useful models for future investigations.

The reduction in cartilage thickness observed in the organ culture tissue sections, following 17β-estradiol [10^{-8} M] supplementation may be directly related to the inhibitory effects of estrogen on chondrocyte proliferation, represented as a decrease in the MI. This type of inhibitory activity for estrogen on cellular proliferation has been previously reported by other investigators.[3,4,22,23] Interestingly, after 2 days in culture, the estrogen-supplemented condylar cul-

Table 2. MEAN MITOTIC INDEX (%) ± SEM

Group	No. of Mitotic Cells	Total No. of Cells	Mitotic Index (%)	Mean MI ± SEM
Control day 2	311	840	37	40.3 ± 1.8
	357	870	41	
	382	895	43	
17β-E_2 day 2	276	881	31	29.7 ± 1.3*
	236	886	27	
	331	1,076	31	
Control day 4	283	885	32	32.0 ± 1.2
	238	793	30	
	207	602	34	
17β-E_2 day 4	142	746	79	18.7 ± 0.3*
	134	703	19	
	126	698	18	

NOTE. Histologic sections from the mandibular condyles in organ culture were analyzed using the Optimas software (Bioscan, Edwards, WA) for the effects of 17β-estradiol [10^{8}M] supplementation on the number of proliferating chondrocytes in each section. Data represent values calculated by the software for the number of BrdU-labeled (column 2) versus total number (column 3) of chondrocytes in each section and calculated mean mitotic index ± standard error of the mean (MI ± SEM) (column 4) for each section on experimental days 2 and 4. Estrogen supplementation resulted in a significant difference in the overall mitotic index between the control and estrogen-supplemented groups as early as experimental day 2 under organ culture conditions. This significant difference persisted for the later experimental time interval.

*Significant difference from controls ($P < .05$).

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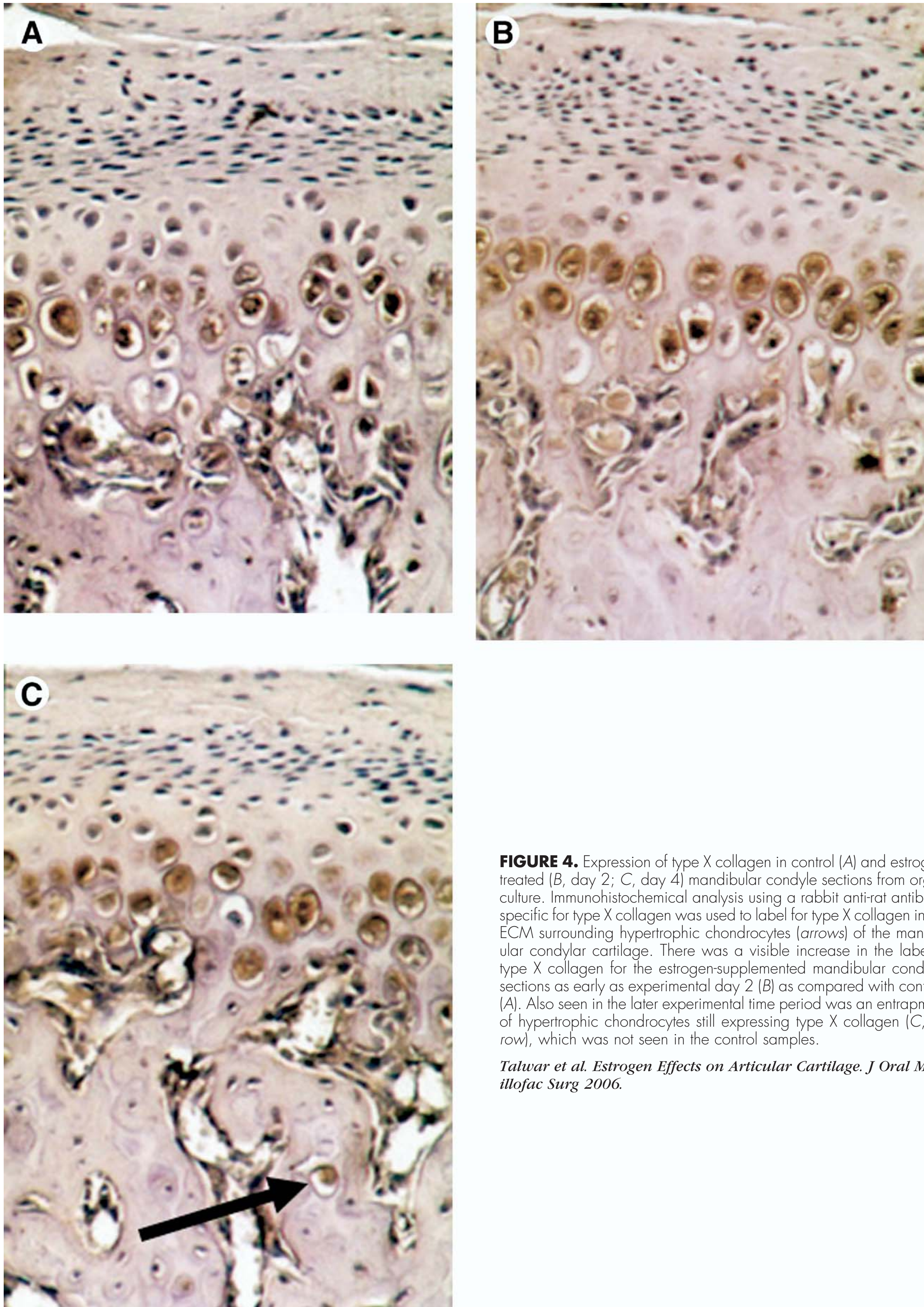

FIGURE 4. Expression of type X collagen in control (*A*) and estrogen-treated (*B*, day 2; *C*, day 4) mandibular condyle sections from organ culture. Immunohistochemical analysis using a rabbit anti-rat antibody specific for type X collagen was used to label for type X collagen in the ECM surrounding hypertrophic chondrocytes (*arrows*) of the mandibular condylar cartilage. There was a visible increase in the labeled type X collagen for the estrogen-supplemented mandibular condylar sections as early as experimental day 2 (*B*) as compared with controls (*A*). Also seen in the later experimental time period was an entrapment of hypertrophic chondrocytes still expressing type X collagen (*C, arrow*), which was not seen in the control samples.

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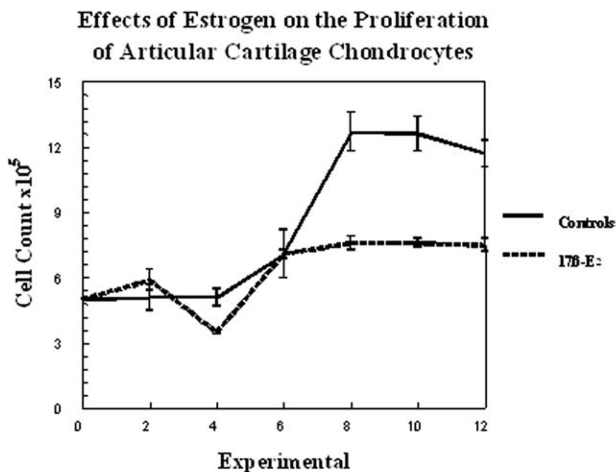

FIGURE 5. Effects of 17β-estradiol [10^{-8} M] supplementation (17β-E_2) on chondrocyte proliferation in cell culture. Chondrocytes isolated from condylar cartilage were plated into tissue culture-treated dishes at an initial concentration of 5 × 10^5 cells per 35 mm plate. Cells were allowed to grow to confluence in a humidified chamber at 37°C, 5% CO_2. On each 2-day experimental time point, cell counts were obtained for control (—) and 17β-estradiol [10^{-8} M] supplementation (–) groups. Estrogen caused a significant decrease in the number of proliferating chondrocytes during logarithmic growth (days 4 to 8) and at confluence (> day 8). *Significant difference ($P < .05$).

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tures also showed a significant reduction in thickness of the hypertrophic zone, with an observable decrease in the number of hypertrophic chondrocytes within that zone, as observed in the histologic section. This reduction in hypertrophic chondrocytes may be secondary to the decreased number of proliferating cells undergoing maturation as a result of estrogen inhibition. After 4 days in culture there was an observable decrease in the number of cells in all of the cartilaginous zones, indicating a continued inhibitory effect of estrogen on chondrocyte proliferation with fewer cells undergoing differentiation and maturation. Although there were fewer chondrocytes in the thinning hypertrophic zone after 4 days in culture, there was an observable increase in the detection of type X collagen for the estrogen-supplemented group. This apparent increase in collagen synthesis may be a result of an increased rate of cellular maturation of the available chondrocytes and an ultimate replacement with bone. Some of the histologic sections, in the later time interval, showed entrapped hypertrophic chondrocytes within the mineralized matrix, which were positively stained for type X collagen in their adjacent extracellular environment. Other investigators have also shown a stimulatory effect on proteoglycan and collagen synthesis by chondrocytes following 17β-estradiol stimulation.[4,14,23,24] These findings provide evidence that the observed thinning of condylar cartilage secondary to 17β-estradiol supplementation is initially related to an inhibition of chondrocyte proliferation. As a result, there may be a consecutive change in the extracellular environment conducive to a stimulatory effect on the rate of chondrocyte maturation and replacement by bone.

In the cell culture system, 17β-estradiol had an inhibitory effect on chondrocyte proliferation, with a significant difference observed between the experimental and control groups during early logarithmic growth and at confluence. These finding were supported by the organ culture study and are, therefore, a good indicator of the inhibitory effects of 17β-estradiol on chondrocyte proliferation. Overall, the results of both in vitro studies suggested that 17β-estradiol [10^{-8} M] had an inhibitory effect on chondrocyte proliferation, while the organ culture study showed increased type X collagen in a thinning hypertrophic chondrocyte zone. Together, these findings suggest that 17β-estradiol supplementation of skeletally mature condylar cartilage chondrocytes may influence the rate of chondrocyte differentiation and maturation by influencing the transition of the proliferative and prechondroblastic cells toward maturity.

The importance of estrogen on the regulation of bone morphology, bone growth, and skeletal maturation has been well documented.[5,25-29] Much of this literature has been focused on the influence of estrogen and other sex hormones on the epiphyseal growth plate of the long bones. During pubertal growth, estrogen and other sex hormones interact with growth hormone to control growth, closure of the epiphyseal plate, and deposition of new osteoid with its subsequent mineralization.[14,25,30-32] Once growth is complete the action of estrogen in the skeletally mature adult is to maintain bone mass. Estrogen deficiency is associated with accelerated bone loss in postmenopausal women; estrogens also play a major role in the regulation of cartilage growth.[33-36]

Recently, there has been greater attention directed toward the effects of reduced estrogen levels, such as is seen during menopause on the histomorphometric and ECM changes in articular cartilage.[15,16,37] In vitro studies on the effects of estrogen on bone and cartilage have been found to be diverse. In chondrocyte cell cultures derived from the growth plate cartilage, 17β-estradiol inhibits cell proliferation and stimulates protein production measure by RNA synthesis, which is highly dependent on the concentration and model being tested.[3,14] The direct effects of estrogen on chondrocytes are mediated through specific estrogen receptors (ERα and ERβ), which have more recently been detected in condylar cartilage from the femoral and mandibular condyles.[16,38] In a chondrocyte cell culture model from the rat costochondral cartilage,

Nasatzky et al[14] demonstrated the stimulatory effects of estrogen on chondrocyte differentiation and matrix protein production. In our investigation, cellular proliferation was significantly inhibited by 17β-estradiol at a concentration of 10^{-8} M in both in vitro systems, with an observable decrease in overall thickness and composition of ECM. As a result of this modulation in the ECM protein composition, 17β-estradiol may be playing a critical role in modulating chondrogenesis.

The maintenance of articular cartilage throughout the life of an organism plays a crucial role in establishing the range of biological adaptability of the cartilage. Modulation of the thickness of the cartilage and the nature of the ECM by estrogen could result in an environment more susceptible to degenerative changes. Although estrogen has been shown to protect against osteoporosis in postmenopausal women, the incidence of degenerative joint disease has been shown to increase under these conditions.[39] The exact role of estrogen in the pathogenesis of osteoarthritis remains controversial, yet most in vivo and in vitro studies support the idea that estrogen is detrimental to cartilage.[40] Much of the research to date addressing the effects of estrogen on chondrocyte proliferation and maturation has been limited to embryonic, postnatal, and pubertal growth. Our study has provided evidence for the inhibitory effects of 17β-estradiol supplementation in skeletally mature cartilage, hence providing 2 additional in vitro models for future experimental analysis.

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