Effects of estrogen on growth plate senescence and epiphyseal fusion

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Estrogen is critical for epiphyseal fusion in both young men and women. In this study, we explored the cellular mechanisms by which estrogen causes this phenomenon. Juvenile ovariectomized female rabbits received either 70 μg/kg estradiol cypionate or vehicle i.m. once a week. Growth plates from the proximal tibia, distal tibia, and distal femur were analyzed after 2, 4, 6, or 8 weeks of treatment. In vehicle-treated animals, there was a gradual senescent decline in tibial growth rate, rate of chondrocyte proliferation, growth plate height, number of proliferative chondrocytes, number of hypertrophic chondrocytes, size of terminal hypertrophic chondrocytes, and column density. Estrogen treatment accelerated the senescent decline in all of these parameters. In senescent growth plates, epiphyseal fusion was observed to be an abrupt event in which all remaining chondrocytes were rapidly replaced by bone elements. Fusion occurred when the rate of chondrocyte proliferation approached zero. Estrogen caused this proliferative exhaustion and fusion to occur earlier. Our data suggest that (*i*) epiphyseal fusion is triggered when the proliferative potential of growth plate chondrocytes is exhausted; and (*ii*) estrogen does not induce growth plate ossification directly; instead, estrogen accelerates the programmed senescence of the growth plate, thus causing earlier proliferative exhaustion and consequently earlier fusion.

In mammals, longitudinal bone growth occurs at the growth plate by endochondral bone formation. The growth plate consists of three principal zones: resting, proliferative, and hypertrophic. The resting zone lies adjacent to the epiphyseal bone and contains infrequently dividing chondrocytes. The proliferative zone contains replicating chondrocytes arranged in columns parallel to the long axis of the bone. The proliferative chondrocytes located farthest from the resting zone stop replicating and enlarge to become hypertrophic chondrocytes (1). These terminally differentiated cells maintain a columnar alignment in the hypertrophic zone. The processes of chondrocyte proliferation, hypertrophy, and cartilage matrix secretion result in chondrogenesis. Simultaneously, the metaphyseal border of the growth plate is invaded by blood vessels and bone cell precursors that remodel the newly formed cartilage into bone (1). The synchronized processes of chondrogenesis and cartilage ossification lead to longitudinal bone growth.

With increasing age, the growth plate undergoes structural and functional changes. The rate of longitudinal bone growth decreases, in large part, because of a decline in chondrocyte proliferation (2–6). These functional senescent changes are accompanied by structural senescent changes. There is a gradual decline in the overall growth plate height (7), proliferative zone height (3), hypertrophic zone height (2), size of hypertrophic chondrocytes (2, 6, 8), and column density (9).

In some mammals, including humans, the growth plate is resorbed at the time of sexual maturation. This process, epiphyseal fusion, terminates longitudinal bone growth. Estrogen is pivotal for epiphyseal fusion in both young men and women (10). This key role for estrogen was confirmed only recently with the recognition of two genetic disorders, estrogen deficiency due to mutations in the aromatase gene (11) and estrogen resistance due to mutations in the estrogen receptor-α gene (12). In both conditions, the growth plate fails to fuse and growth persists into adulthood. Conversely, premature estrogen exposure, e.g., precocious puberty, leads to premature epiphyseal fusion (13).

The mechanism by which estrogen promotes epiphyseal fusion is not known. Previous reports suggest that estrogen accelerates growth plate ossification by stimulating vascular and bone cell invasion of the growth plate cartilage, causing ossification to advance beyond the hypertrophic zone into the proliferative and resting zones (14–16). This proposed mechanism of estrogen action would be expected to induce epiphyseal fusion promptly, a prediction that does not match clinical experience. Prompt fusion occurs only in estrogen-deficient adults treated with estrogen (10, 17, 18). In children, epiphyseal fusion occurs only after years of estrogen exposure. The accelerated ossification hypothesis does not readily explain this delayed action.

The current study was designed to explore the underlying cellular mechanisms by which estrogen causes growth plate fusion. Because this process involves chondrocytes, osteoblasts, osteoclasts, and endothelial cells interacting within the complex structure of the growth plate, an *in vivo* model was chosen. Rabbits were selected for this study because rabbits, like humans but unlike rodents, fuse their epiphyses at the time of sexual maturation and in response to sex steroids (19–21). We also used physiological doses of estradiol and initiated treatment at the expected age of onset of sexual maturation (22) to mimic physiological conditions.

Materials and Methods

Animals. Forty New Zealand White female rabbits were ovariectomized at 12 weeks of age (Covance Research Products, Denver, PA). All animals received National Institutes of Health open formula rabbit ration (NIH 32) and water ad libitum. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (23). The protocol was approved by the Animal Care and Use Committee (National Institute of Child Health and Human Development, National Institutes of Health).

Study Design. Beginning at 16 weeks of age, animals received weekly i.m. injections of either 70 μg/kg estradiol (E_2) cypionate or vehicle (cottonseed oil). After 2, 4, 6, or 8 weeks of treatment (7 days after the last injection with E_2 cypionate or vehicle), groups of animals ($n = 5$ per treatment group per time point) were killed by pentobarbital overdose. BrdUrd (50 mg/kg i.p., Sigma) was administered 7 h and again 2 h before the animals were killed. Femurs and tibiae were preserved for histological analysis. Blood was drawn from five of the E_2 cypionate-treated and five of the vehicle-treated rabbits 2 days after the second injection to assess peak E_2 levels and in all animals just before sacrifice for measurement of E_2, estrone (E_1), and insulin-like

Abbreviations: E_2, estradiol; E_1, estrone; IGF-I, insulin-like growth factor I; PT, proximal tibia; DT, distal tibia; DF, distal femur; AUC, area under the curve.

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growth factor I (IGF-I). For comparison, plasma E_2, E_1, and IGF-I levels were also measured weekly for 4 weeks in three intact, untreated 5-month-old New Zealand White female rabbits.

Growth Rate Determination. Radiographs of both tibiae were obtained on day 0 (before the first injection) and repeated every 2 weeks. For each radiograph, animals were sedated and positioned on a horizontal cassette containing x-ray film, with the hind leg of interest placed on the cassette. Using these radiographs, the maximal length of the tibia was measured by using a digital vernier caliper (24). We averaged both tibial lengths for individual animals at each time point and calculated the growth velocity at 2-week intervals.

Tissue Processing. Tibiae and femurs were fixed in 10% phosphate-buffered formalin for 48 h, placed in 70% ethanol for storage, decalcified in formic acid, and embedded in paraffin. Sagittal 5-μm sections of the distal femoral (DF), proximal tibial (PT), and distal tibial (DT) growth plates were obtained from the center of the bone.

BrdUrd Immunohistochemistry. BrdUrd is incorporated into newly synthesized DNA and thus labels replicating cells. This method can be used to assess cell proliferation *in vivo* in many tissues, including the growth plate (25). Labeled cells were visualized by immunohistochemical staining for BrdUrd by using a commercial kit containing biotinylated monoclonal mouse anti-BrdUrd (HCS24, Oncogene Research Products, Cambridge, MA). BrdUrd staining was performed as per the manufacturer's protocol except that the anti-BrdUrd antibody was diluted 1:1 with 1 N PBS. Labeled and unlabeled cells were counted in ≈20 chondrocyte columns near the center of each growth plate section. Per convention (3), the proliferative zone chondrocyte closest to the resting zone was considered to be in position 1. Columns were included in the analysis only if this topmost cell was included in the plane of the section, thus permitting us to identify the position of other cells in that column.

Cell Kinetics. The BrdUrd-labeling index was defined as the number of BrdUrd-labeled cells at a specific cell position divided by the total number of labeled and unlabeled cells at that position. The calculation of the labeling index for a specific cell position required an assessment of at least five cells in that position. The BrdUrd–area under the curve (AUC) was calculated as the sum of labeling indices over all positions in the columns. The BrdUrd–AUC reflects the average number of labeled cells per column and therefore the average chondrocyte proliferation rate per column.

Quantitative Histology. Histological evaluations were performed on Masson Trichrome-stained tissue sections by using a light microscope with a VIA-100 video measurement system (Boeckeler, Tucson, AZ). Growth plates were considered to be fused if 50% or more of the total length of the growth plate cartilage was replaced by bone. In partially (<50%) fused growth plates, measurements were taken in unfused areas only. All histological measurements were performed in the central two-thirds of the growth plate sections. Heights were measured parallel to the chondrocyte columns. Column density was calculated as the number of columns per 1-mm growth plate width. We assessed growth plate height and column density in three areas of each growth plate and averaged the results. The number of proliferative and hypertrophic cells was counted in ≈15 intact columns per growth plate, and the counts for each cell type were averaged for individual growth plates. For this analysis, hypertrophic chondrocytes were operationally defined by a height ≥10 μm (26). The terminal hypertrophic chondrocyte was defined as the cell in the last lacuna that was not invaded by metaphyseal blood vessels. The height of this cell was measured in 25 different columns per growth plate and averaged.

Assays. Plasma E_1, E_2, and IGF-I levels were determined at Endocrine Sciences (Calabasas Hills, CA) by RIA.

Statistics. All data are expressed as mean ± SEM. One animal in the 8-week vehicle-treated group was excluded from analysis due to markedly abnormal growth (tibial growth −4.5 SD for the control group). The overall effects of time and treatment in each growth plate location were evaluated by two-way ANOVA. Because of the limited sample size at each time point, we did not perform pairwise comparisons between treatment groups. Plasma hormone levels were compared by Student's t test. For the relationship between time and BrdUrd–AUC, the least square regression line was determined. The X-intercept of this line was used as an estimate of the time of proliferative exhaustion (proliferation rate = 0). Median time of fusion was determined by linear interpolation. Quantitative histological and immunohistochemical data from completely fused growth plates were not included in the statistical analysis because we wanted to study effects on growth plate chondrocytes before fusion. Inclusion of these data would have accentuated the effects of time and treatment and thus increased statistical significance. The results on the DT epiphyses are presented graphically for comparison but were excluded from analysis because of paucity of data due to fusion at an early time point.

Results

Timing of Epiphyseal Fusion. In the vehicle-treated animals, the timing of fusion varied among the growth plates studied. The DT growth plates fused between 2 and 6 weeks of treatment, whereas the DF and PT growth plates remained open at the end of the experiment (Fig. 1). Estrogen treatment advanced fusion at all three sites (PT, $P < 0.05$; DF, $P < 0.001$; DT, $P < 0.05$) (Fig. 1).

Growth Rate and Weight Gain. Weight and tibial length were similar in both groups at initiation of treatment (2611 ± 33 g vs. 2612 ± 28 g; 104.9 ± 0.5 mm vs. 105.8 ± 0.4 mm; estrogen vs. vehicle, respectively). In vehicle-treated animals, the tibial growth rate declined with age ($P < 0.001$, Fig. 2*A*). Estrogen treatment accelerated this decline ($P < 0.001$, Fig. 2*A*). Estrogen treatment caused this suppression of linear growth despite accelerated weight gain ($P < 0.001$, Fig. 2*B*).

Cell Kinetics. In the PT growth plate, the rate of chondrocyte proliferation, as assessed by the BrdUrd-labeling index, was greatest in the upper third of the proliferative zone (i.e., the portion of the column closest to the epiphysis, Fig. 3). In vehicle-treated animals, the rate of chondrocyte proliferation decreased with age. Estrogen treatment accelerated this decline (Fig. 3). A similar pattern was observed in the DF growth plates (data not shown).

The area under each curve in Fig. 3 was calculated by summing the labeling indices at every position in the column. This sum (BrdUrd–AUC) reflects the proliferation rate per column. In the vehicle-treated group, the proliferation rate per column declined with age (PT, $P < 0.001$; DF, $P < 0.001$; Fig. 4 *A–C*). Estrogen accelerated this decline (PT, $P < 0.001$; DF, $P < 0.01$; Fig. 4 *A–C*). As a result, the proliferation rate approached zero earlier in E_2 cypionate-treated than in vehicle-treated animals. The timing of this event, which we term proliferative exhaustion, appeared to correlate with the timing of epiphyseal fusion (Fig. 4 *D* and *E*). This temporal relationship was observed at all growth plate sites and in both treatment groups (Fig. 4 *D* and *E*).

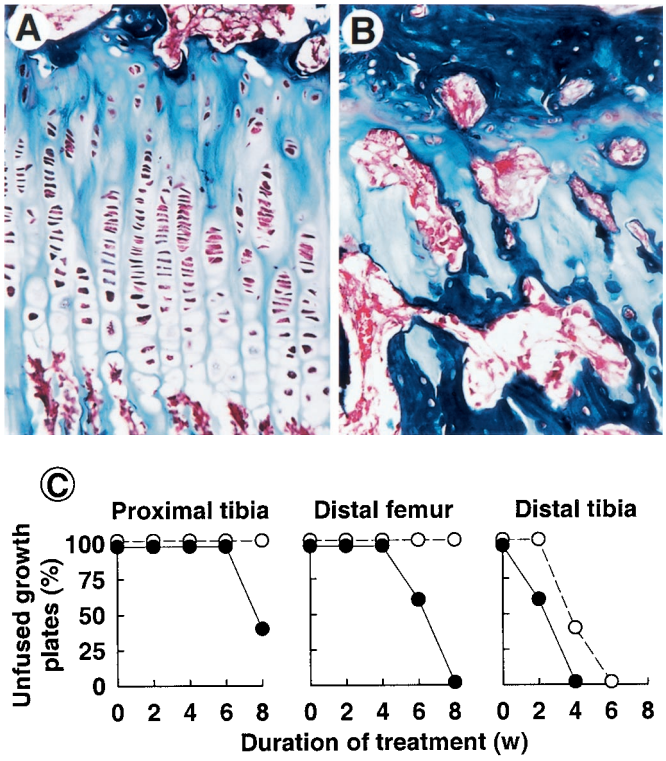

Fig. 1. Effect of estrogen treatment on the timing of epiphyseal fusion. Ovariectomized rabbits were treated with either i.m. E_2 cypionate (●) or vehicle (○) beginning at age 16 weeks. Epiphyseal fusion was evaluated histologically at three anatomical sites by using sagittal, Masson Trichrome-stained sections. (*A*) Representative unfused proximal tibial growth plate after 8 weeks of vehicle treatment. Chondrocyte columns are intact. (*B*) Representative fused proximal tibial growth plate after 8 weeks of estrogen treatment. Chondrocyte columns have been replaced by vascular and bone tissue. (*C*) Values indicate the percent of animals with unfused growth plates. Growth plates were considered to be fused if 50% or more of the total length of the growth plate cartilage was replaced by bone.

Quantitative Histology. In the vehicle-treated rabbits, with time, there was a significant decline in the total growth plate height (PT, $P = 0.001$; DF, $P = 0.01$; Fig. 5*A*), the number of proliferative chondrocytes per column (PT, $P < 0.001$; DF, $P < 0.001$; Fig. 5*B*), the number of hypertrophic chondrocytes per column (PT, $P < 0.05$; DF, $P < 0.01$; Fig. 5*C*), the height of the terminal hypertrophic chondrocyte (PT, $P < 0.001$; DF, $P < 0.001$; Fig. 5*D*), and the column density (PT, $P < 0.01$; DF, $P < 0.001$; Fig. 5*E*). Estrogen treatment accelerated the decline in all of these parameters: the total growth plate height (PT, $P < 0.05$; DF, $P < 0.05$; Fig. 5*A*), the number of proliferative chondrocytes per column (PT, $P < 0.001$; DF, $P < 0.001$; Fig. 5*B*), the number of hypertrophic chondrocytes per column (PT, $P < 0.05$; DF, $P < 0.001$; Fig. 5*C*), the height of the terminal hypertrophic chondrocyte (PT, $P < 0.01$; DF, $P = 0.09$; Fig. 5*D*), and the column density (PT, $P < 0.01$; DF, $P < 0.001$; Fig. 5*E*).

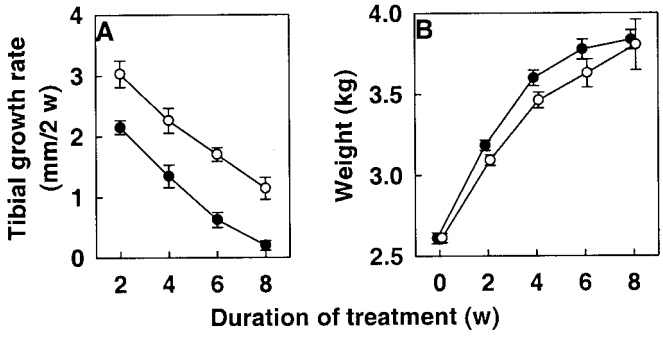

Fig. 2. Effect of estrogen treatment on mean (±SEM) tibial growth rate (*A*) and body weight (*B*). Ovariectomized rabbits were treated with either i.m. E_2 cypionate (●) or vehicle (○) beginning at age 16 weeks. Tibial length was determined from radiographs obtained every 2 weeks.

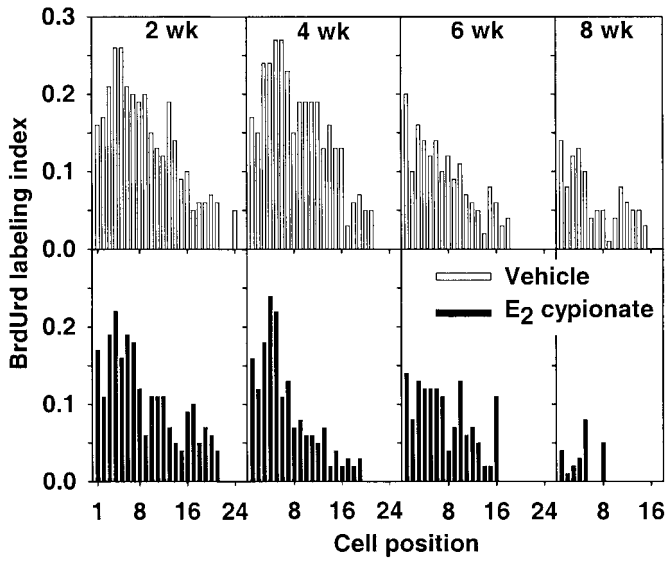

Fig. 3. BrdUrd-labeling profile in the proximal tibial growth plate. Ovariectomized rabbits were treated with either i.m. E_2 cypionate (*Lower*) or vehicle (*Upper*) beginning at age 16 weeks. Proliferating chondrocytes were detected by BrdUrd immunohistochemistry. BrdUrd-labeling indices (BrdUrd-positive cells/total cells) were calculated for every cell position in the column, and the results were averaged for each treatment group. Cell position one denotes the proliferative chondrocyte closest to the resting zone.

Epiphyseal fusion appeared to be a rapid event. Within a single treatment group and at a single anatomical site, growth plates usually were either completely fused, with no remaining chondrocyte columns or were completely unfused (Fig. 6). Thus, near the time of fusion, some of the histological parameters appeared to show a bimodal distribution; the fused growth plates showed values of zero, whereas the unfused growth plates showed values similar to preceding time points (Fig. 6 *Center* and *Bottom*). This bimodal distribution was not clearly seen for the number of proliferative chondrocytes (Fig. 6 *Top*).

Plasma Hormone Levels. In the vehicle group, all plasma E_2 levels were below the detection limit of the assay (<5 pg/ml; to convert to pmol/liter, multiply by 3.671), whereas the E_2 cypionate group had E_2 concentrations (peak 140 ± 9, trough 27 ± 3 pg/ml) comparable with intact, untreated animals (59 ± 34 pg/ml). E_1 levels were similar for E_2 cypionate (28 ± 2 pg/ml; to convert to pmol/liter, multiply by 3.699) and vehicle-treated (24 ± 4 pg/ml) animals. IGF-I concentrations were higher ($P < 0.05$) in E_2 cypionate (210 ± 12 ng/ml) than in vehicle-treated animals (174 ± 10 ng/ml) and similar to intact animals (196 ± 14 ng/ml).

Discussion

Growth plates from control animals underwent senescent changes in function and structure. With age, there was a progressive decline in the longitudinal growth rate due, in part, to a decline in the rate of chondrocyte proliferation. This progressive loss of function was accompanied by a gradual decline in growth plate height, the number and size of chondrocytes present in the growth plate, and column density. Some of the

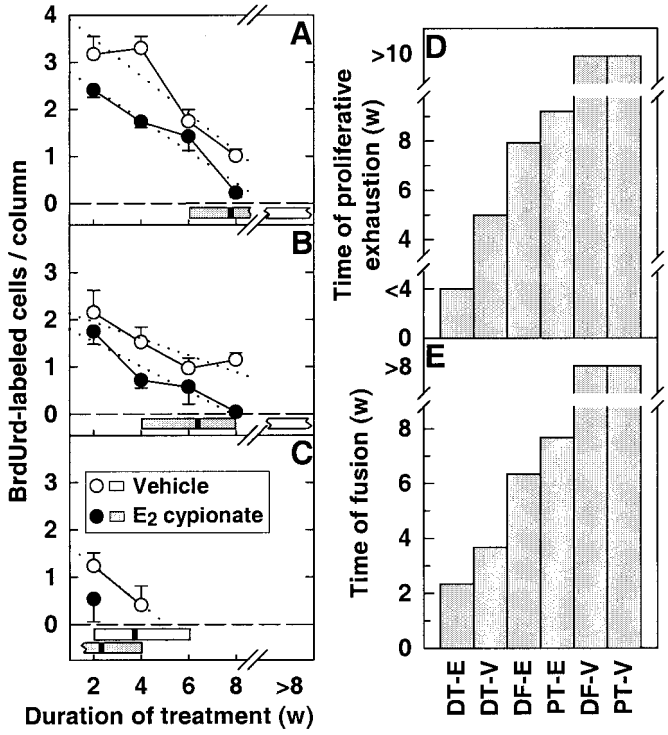

Fig. 4. (*A–C*) Effect of estrogen treatment on the mean (±SEM) chondrocyte proliferation rate per cell column. Ovariectomized rabbits were treated with either i.m. E_2 cypionate (●) or vehicle (○) beginning at age 16 weeks. Proliferating chondrocytes were detected by BrdUrd immunohistochemistry. The average number of labeled cells per column (BrdUrd–AUC) was calculated as the sum of labeling indices over all cell positions in the columns in the proximal tibia (*A*), distal femur (*B*), and distal tibia (*C*). Dotted lines indicate regression lines. Horizontal bars indicate the time range of epiphyseal fusion (beginning at the time point when fusion was first observed and ending at the time point when all growth plates were fused); black lines within the bars represent the median time of fusion (50% of growth plates fused, determined by interpolation from data in Fig. 1). (*D* and *E*) Relationship between time of proliferative exhaustion and time of epiphyseal fusion. The time of proliferative exhaustion was estimated by using the X-intercept of the regression lines shown in *A–C*. Median time of fusion was determined by linear interpolation by using the data shown in Fig. 1. E, E_2 cypionate-treated; V, vehicle-treated.

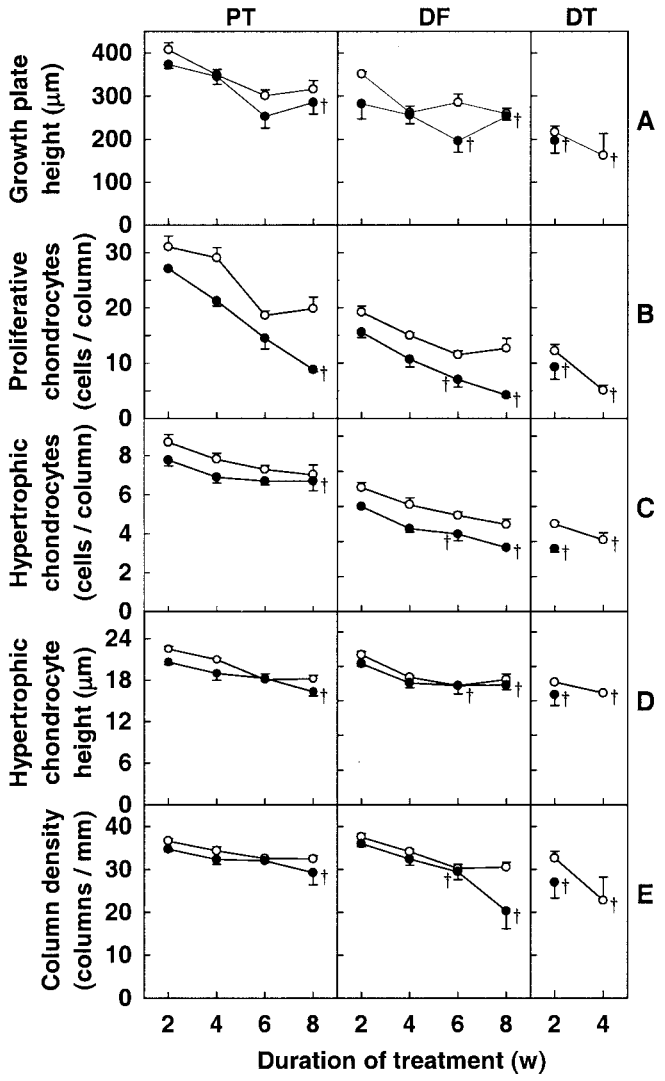

Fig. 5. Effect of estrogen on growth plate structure. Ovariectomized rabbits were treated with either i.m. E_2 cypionate (●) or vehicle (○) beginning at age 16 weeks. Quantitative histology (mean ± SEM) was performed on sagittal, Masson Trichrome-stained sections by using a video measurement system. Heights were measured parallel to the chondrocyte columns. (*C*) Hypertrophic chondrocytes were operationally defined by a height of at least 10 μm. (*D*) Cell height was measured in the terminal hypertrophic chondrocyte, the chondrocyte located closest to the metaphysis. (*E*) Column density = the number of chondrocyte columns per 1-mm growth plate width. †, growth plates completely or nearly (>50%) fused were excluded from the analysis.

observed structural changes, such as decreased number of proliferative and hypertrophic chondrocytes, might be secondary to the decline in chondrocyte proliferation. A similar process occurs in other mammals including humans (2–9). Based on growth plate transplantation experiments, the senescent decline in growth rate appears not to be due to a hormonal or other systemic mechanism but rather to a mechanism intrinsic to the growth plate (27). Indirect evidence suggests that growth plate chondrocytes have a finite proliferative capacity that is gradually exhausted, causing growth to slow and eventually halt (28, 29).

Estrogen treatment did not appear to induce any qualitative changes in the growth plate; however, it did accelerate the senescent decline in all functional and structural markers studied. These effects did not appear to be due to decreased nutritional intake or to suppression of the growth hormone–IGF-I axis. To the contrary, estrogen treatment accelerated weight gain and increased serum IGF-I levels, suggesting that estrogen acts directly on the growth plate.

Epiphyseal fusion appeared to be an abrupt event. Growth plates usually were observed to be either completely fused, with no remaining chondrocyte columns, or completely unfused with well maintained columns, suggesting that fusion is a rapid process in which all remaining chondrocytes are quickly replaced by vascular and bone cells. The timing of epiphyseal fusion varied among anatomical sites, occurring earlier in the distal tibia than in the proximal tibia or distal femur. A similar sequence occurs in intact rabbits (30, 31).

Estrogen treatment caused fusion to occur earlier at all three anatomical sites studied. That estrogen accelerated both senescence and fusion suggests that these two processes are linked; fusion may be triggered when the senescence program advances to a certain critical point. Thus, estrogen may not stimulate fusion directly. Instead, it may simply accelerate the senescence program, thus secondarily triggering fusion earlier. In particular, fusion occurred at approximately the same time that the proliferation rate approached zero. This temporal association, which was observed at all anatomical sites studied and in both treatment groups, suggests that fusion is triggered when the prolif-

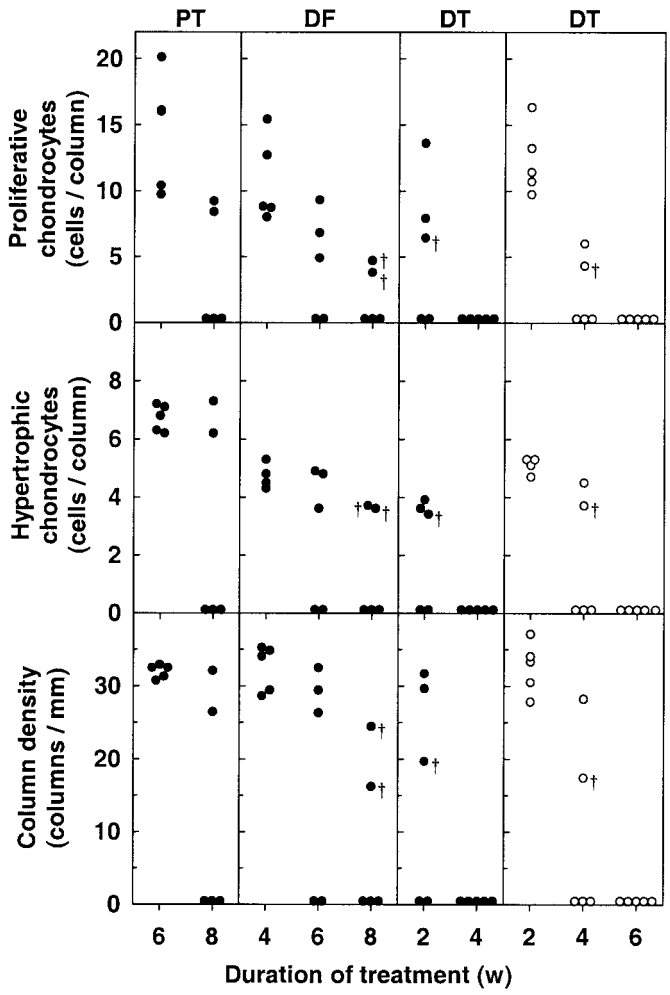

Fig. 6. Growth plate structure around the time of epiphyseal fusion. Only data from growth plate sites that underwent fusion during the experiment are shown. Ovariectomized rabbits were treated with either i.m. E_2 cypionate (●) or vehicle (○) beginning at age 16 weeks. Quantitative histology was performed on sagittal, Masson Trichrome-stained tissue sections by using a video measurement system. Hypertrophic chondrocytes were operationally defined by a height ≥10 μm. Column density = the number of columns per 1-mm growth plate width. Symbols indicate data from individual animals. †, growth plates with partial (<50%) fusion. All measurements were obtained from unfused areas. Growth plates completely or nearly (>50%) fused were excluded from the analysis.

erative potential of the growth plate chondrocytes is finally exhausted. Estrogen, by accelerating senescence, may hasten proliferative exhaustion and thus cause earlier fusion. This model would explain why estrogen affected growth plate function and structure promptly, whereas the effect on fusion was more delayed.

Our data do not support the hypothesis that estrogen promotes fusion by directly accelerating vascular and bony invasion of the growth plate. Accelerated invasion would lead to elimination of the hypertrophic zone first, followed by the proliferative and then the resting zone. To the contrary, we found that the hypertrophic zone was well maintained through weeks of estrogen therapy, even just before fusion. Several previous studies do suggest that estrogen might accelerate ossification, but they do not clearly define the underlying mechanism (14–16). Unlike the current study, these earlier studies primarily used rodents, which do not undergo fusion at the time of sexual maturation or in response to exogenous estrogen (19). In addition, the estrogen doses in these studies were either supraphysiological or poorly quantitated, or the histological effects were not well described. Although our data suggest that estrogen promotes fusion indirectly by accelerating growth plate senescence, the findings do not exclude the possibility that, in senescent growth plates, estrogen is also required to complete the process of fusion.

Estrogen also regulates human bone growth. In humans, as in rabbits, high doses of estrogen suppress growth (32). However, lower concentrations accelerate human growth (33), accounting, at least in part, for the pubertal growth spurt in both boys and girls (34). This growth-promoting effect of estrogen seems to be due in part to stimulation of the growth hormone–IGF-1 axis and in part to a growth hormone-independent mechanism (34). In rabbits, a growth-promoting effect of estrogen is not well documented (30).

Despite this difference, certain clinical observations suggest that estrogen accelerates growth plate senescence in humans as it does in our animal model. The first such clinical observation is that estrogen accelerates bone age advancement. The bone age is a radiological measure of skeletal maturation, which is inversely related to the amount of linear growth remaining for an individual child (35). Therefore, the bone age can be considered a surrogate marker for growth plate senescence. Thus, the fact that estrogen accelerates bone age advancement suggests that estrogen accelerates the growth plate senescence program in humans.

A second clinical observation consistent with our model is that children who are exposed to estrogen because of precocious puberty and who are treated with a gonadotropin-releasing hormone analog to suppress puberty often show a subnormal linear growth rate. Endocrinological evaluation of these children has not revealed a clear hormonal explanation (36, 37). Our model provides an explanation for this phenomenon; the low growth rate may be due to excessive growth plate senescence induced by the previous estrogen exposure.

A third clinical observation consistent with our model is that the duration of estrogen exposure required to induce epiphyseal fusion depends on age. Young children with untreated precocious puberty do not fuse for many years. At the other extreme, two adult men with unfused epiphyses due to aromatase (and hence estrogen) deficiency showed fusion within 6 to 9 months of starting estrogen therapy (10, 17, 18). Our model provides a possible explanation for these observations. The growth plates of young children are less senescent and thus require prolonged estrogen exposure to drive the senescence program to completion. The growth plates of older individuals are more senescent and therefore require a more brief exposure.

Previous studies of cell kinetics suggest that the growth plate contains stem-like cells either in the resting zone or in the uppermost part of the proliferative zone (3, 9). Based on indirect evidence, we previously proposed that senescence is a function of the number of replications that the stem-like cells have undergone (28, 38). The stem-like cells may have a finite proliferative potential (and thus are not true stem cells) that is gradually exhausted, causing growth to decelerate with age. If this model is correct, then estrogen might act by accelerating replicative senescence of the stem-like cell, causing earlier exhaustion of its proliferative potential. In other cell types, replicative senescence is associated with a reduction in telomere length (39). Thus, estrogen might act on the growth plate by accelerating telomere shortening. However, telomerase-deficient mice show decreased telomere length with each successive generation but do not exhibit striking growth abnormalities (40). Replicative senescence also may involve the increased expression of genes that inhibit progression through the cell cycle, for example p53 or p21 (39). Thus, estrogen might act by directly increasing expression of these inhibitors.

Estrogen is not the only factor that influences the timing of epiphyseal fusion. Other factors, such as growth hormone, thyroid hormone, and glucocorticoids appear to affect skeletal maturation in humans (38). In addition, retinoid (41) and warfarin (42) toxicity can cause premature fusion. It is not clear whether these conditions cause ossification directly or indirectly by accelerating growth plate senescence.

In conclusion, our data suggest the following new model. (*i*) The growth plate undergoes programmed senescence including a decline in growth rate, proliferation rate, and the number and size of chondrocytes. This program may be due to replicative senescence of the growth plate chondrocytes. (*ii*) Epiphyseal fusion is a rapid process that is triggered when the senescence program reaches a critical point. Specifically, epiphyseal fusion seems to be triggered when the proliferative potential of the growth plate chondrocytes is finally exhausted. (*iii*) Estrogen accelerates the growth plate senescence program, thereby exhausting the proliferative potential of growth plate chondrocytes sooner, consequently triggering earlier epiphyseal fusion. This model may also apply to humans, providing an explanation for certain clinical observations: the catch-down growth in children following estrogen exposure, the prolonged and age-dependent delay between the onset of estrogen and the onset of fusion, and the acceleration of bone age by estrogen.

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