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Age-related changes in human oestrogen receptor α function and levels in osteoblasts

Michael A. ANKROM*[1], Julie A. PATTERSON*[1,2], Patricia Y. d'AVIS*, Ulrich K. VETTER†, Marc R. BLACKMAN‡, Paul D. SPONSELLER§, Matthew TAYBACK*, Pamela Gehron ROBEY‖, Jay R. SHAPIRO* and Neal S. FEDARKO*[3]

*Division of Geriatrics and Gerontology, Department of Medicine, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, U.S.A., †Allgemeines Krankenhaus Heidberg, Tangstedter Landstrasse 400, 22413 Hamburg, Federal Republic of Germany, ‡Division of Endocrinology and Metabolism, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224, U.S.A., §Department of Orthopaedics, Johns Hopkins University School of Medicine, Baltimore, MD 21224, U.S.A., and ‖Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, N.I.H. Bethesda, MD 20892, U.S.A.

Oestrogen receptors (ERs) are present in human osteoblasts and mediate anti-resorptive effects on bone. Human osteoblast-like cells derived from different aged healthy female donors not on hormone replacement therapy were utilized under well-defined conditions *in vitro* to investigate ER function and levels. Treatment with 0.1 nM oestradiol-17β of cell strains derived from eight young women (less than 50 years of age) increased hydroxyproline levels significantly [an average (2.2 ± 0.1 S.E.M.)-fold increase], whereas cells derived from nine older women (more than 50 years of age) were not significantly affected. Similarly, cell strains, derived from younger women, transfected with a consensus oestrogen-responsive element linked to chloramphenicol acetyltransferase exhibited a greater response to oestrogen than strains derived from older women. When basal ERα levels were measured by enzyme immunoassay and normalized on a per cell basis, osteoblast-like strains derived from younger women ($n = 24$) had a mean value of 2.54 ± 0.16 fmol of ERα per 10^6 cells. In contrast, strains derived from older women ($n = 20$) had a mean value of 5.44 ± 0.48 fmol of ERα per 10^6 cells. An age-related increase in ERα number was also observed in human skin-derived fibroblasts and directly in dermal biopsies from women not on hormone replacement therapy. The results demonstrate ligand concentration-dependent ERα induction and indicate a loss of receptor regulation and diminution of ligand–receptor signal transduction with increasing donor age.

INTRODUCTION

Oestrogen signal transduction is mediated through genomic effects of the oestrogen receptor (ER). The ER is a member of the steroid hormone receptor group within the nuclear hormone receptor superfamily, which, when activated, bind specific DNA sequences and regulate the expression of specific genes [1]. The steroid hormone receptors have been shown to act as either trans-activating or repressing factors, exerting either a positive or a negative effect on gene expression [2]. The presence of ER in bone was initially determined by radioimmunoassay with rat and human osteosarcoma cells, and bone from McCune–Albright patients, normal children and patients with breast cancer, and subsequently by [^{3}H]oestradiol nuclear binding assay with normal human osteoblasts [3–7]. Recent studies have described a novel second form of ER (ERβ) that shares a high degree of conservation of the DNA-binding domain, has sequence similarity to the ligand-binding domain of the traditional ER (now termed ERα) [8–11] and is expressed in rat bone [12].

Oestrogen loss by surgical ovariectomy or from natural menopause results in accelerated bone loss in both humans and animals [13–15]. As a result, oestrogen replacement in both peri-menopausal and post-menopausal women has become a mainstay of therapy in women to maintain bone mass and decrease the incidence of bone fractures [16–18]. Endogenous and exogenous oestrogens are believed to modulate bone metabolism by enhancing osteoblast bone formation and inhibiting osteoclast bone resorption, thereby maintaining bone density [19]. Increased osteoclast resorptive activity is believed to arise from the loss of the oestrogen-dependent repression of interleukin 6 levels [20]. The mechanism by which oestrogen increases bone formation has yet to be defined. Age-related changes in oestrogen levels also give rise to changes in the properties of dermal skin; these changes are reversed by hormone replacement therapy [21]. The present study was undertaken to characterize oestrogen responsiveness and ER levels as a function of donor age in osteoblast-like cells and dermal fibroblasts.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), Ca^{2+}-free, Phenol Red-free DMEM, Earle's Minimal Essential Medium, fetal bovine serum (FBS), penicillin and streptomycin were obtained from Biofluids (Rockville, MD, U.S.A.). Collagenase D was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Sodium

Abbreviations used: CAT, chloramphenicol acetyltransferase; CMFH, Ca^{2+}/Mg^{2+}-free Hepes; CV, coefficient of variance; DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunoassay; ER, oestrogen receptor; ERE, oestrogen-responsive element; FBS, fetal bovine serum; tk, thymidylate kinase.

[1] These authors contributed equally to the work.

[2] Present address: Clinical Geriatric Programs, Center for Aging, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756, U.S.A.

[3] To whom correspondence should be addressed (e-mail ndarko@welchlink.welch.jhu.edu).

ascorbate, glutamine and gentamycin were obtained from Gibco-BRL (Gaithersburg, MD, U.S.A.). Fatty-acid free BSA and bovine type I collagen were supplied by ICN (Costa Mesa, CA, U.S.A.). Epidermal growth factor and platelet-derived growth factor were from Calbiochem (San Diego, CA, U.S.A.). Insulin, transferrin, selenium and recombinant human insulin-like growth factor I were obtained from Collaborative Biomedical Products (Bedford, MA, U.S.A.). Oleic acid, linoleic acid, stearic acid, ceruloplasmin and thymidine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pronectin was supplied by Protein Polymer Technologies (San Diego, CA, U.S.A.). Radiolabelled L-[2,3,4,5-^{3}H]proline (114 Ci/mmol) and L-[4,5-^{3}H]leucine (133 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). pCMV-β control vector was from Clonetech Laboratories (Palo Alto, CA, U.S.A.); pA2(-482/-87)tk–CAT (in which tk stands for thymidylate kinase and CAT for chloramphenicol acetyltransferase) and pBL–CAT2 were kindly provided by Dr. G. U. Ryffel (Essen University, Essen, Germany). An enzyme immunoassay (EIA) for oestradiol-17β was supplied by Cayman Chemical (Ann Arbor, MI, U.S.A.) and monoclonal antibody ERα-EIA was obtained from Abbott Laboratories (Chicago, IL, U.S.A.). All other reagents were of the best analytical grade available.

Patients

Recruitment of subjects donating osteoblasts, fibroblasts and dermal biopsies was in accordance with approved procedures of the Institutional Review Board, Johns Hopkins Bayview Medical Center (Baltimore, MD, U.S.A.). Human trabecular osteoblasts were cultured by using a modification of the method of Robey and Termine [22]. Normal bone samples were taken during surgery for fracture non-union and grafting with benign bone tumours. In some cases, samples were obtained during autopsy by sterile biopsy within 2–6 h of death. Extensive metabolic studies of osteoblasts isolated in this fashion have been reported [23,24]. The specimens were minced to the consistency of coarse sand, rinsed free of marrow cells with sterile PBS and digested with 1 mg/ml collagenase D. Bone cells were isolated and cultured in low-Ca^{2+} DMEM containing 10% FBS, 4.5 g/l glucose, 2 mM glutamine, 25 μg/ml ascorbate, 100 i.u./ml penicillin and 100 μg/ml streptomycin. All osteoblast-like strains were studied in primary culture after passage from explant culture. For oestrogen dose–response studies, a 'zero' oestradiol-containing medium was obtained by using the above medium containing charcoal-stripped FBS.

Nine normal human fibroblast cell strains (ages 5, 16, 18, 19, 31, 66, 66, 80 and 82 years, in passage 3–5) were isolated from skin biopsies measuring 4 mm obtained from the upper inner arm obtained from healthy females not on hormone replacement therapy. Eight human fibroblast cell strains from normal female subjects (ages 23, 24, 31, 44, 50, 60, 61 and 71 years) were obtained from the National Institutes on Aging (NIA) Aging Cell Repository maintained by the Coriell Institute for Medical Research (Camden, NJ, U.S.A.). Fibroblasts were cultured in Earle's Minimal Essential Medium containing non-essential amino acids, 10% FBS, 100 i.u./ml penicillin and 100 μg/ml streptomycin.

For direct measurements *in vivo*, dermal biopsies from the lower back of healthy women of ages 34–86, on no sex steroid therapy, were obtained and were stored immediately in liquid nitrogen until analysed. The epidermis as well as the distal adipocyte-enriched dermis were removed and samples were analysed in duplicate by ERα EIA in accordance with the manufacturer's protocol.

Serum-free culture

A serum-free medium that promoted osteoblast proliferation and cell survival for up to 4 weeks was used to culture osteoblasts seeded at a high density (50000 cells/cm^{2}). The basal medium consists of Phenol Red-free, Ca^{2+}-free DMEM with 4.5 g/l glucose containing 1 mg/ml fatty-acid free BSA, 0.2 mM Ca^{2+}, 2 mM glutamine, 10 μg/ml gentamycin, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 10 nM selenium, 2 μg/ml oleic acid, 2 μg/ml linoleic acid, 2 μg/ml stearic acid, 0.05 i.u./ml ceruloplasmin and 0.01 nM thymidine. For enhanced proliferation, the medium was modified to include 1.0 nM recombinant human insulin-like growth factor I and 2 ng/ml platelet-derived growth factor. Ceruloplasmin was 'detoxified' with Pierce's Detoxi-Gel Endotoxin Removing AffinityPak Prepacked columns (Pierce Chemicals, Rockville, IL, U.S.A.). A crucial component of the serum-free culture system was that the bone cells were seeded on tissue culture plates coated with 50 μg/ml type I collagen (bovine) and 1 μg/ml fibronectin (human) or 5 μg/ml pronectin, a polymer incorporating multiple copies of the RGD (Arg-Gly-Asp) cell attachment ligand of human fibronectin interspersed between repeated structural peptide segments.

Collagen analysis

Collagen was determined after steady-state radiolabelling as radioactivity in hydroxyproline by a modification of the method of Schmid and Conrad [25]. Collagen was assayed in cultures labelled with 10 μCi/ml [^{3}H]proline; at the end of an 18 h labelling time the medium was aspirated and the monolayer was rinsed twice with Tris-buffered saline, pH 7.6, with the washes added to the medium and taken as the 'medium pool'. The 'cell layer' pool was generated by extraction with a Tris buffer, pH 7.4, containing 4 M guanidinium chloride, 0.005 M benzamidine, 0.01 M *N*-ethylmaleimide, 0.1 M 6-aminohexanoic acid, 0.001 M PMSF and 2% (v/v) Triton X-100. Aliquots of the original samples were made 70% (v/v) in ethanol, chilled overnight at −20 °C, precipitated by centrifugation at 8000 ***g*** in an Eppendorf Microfuge, resuspended in Tris/HCl buffer, pH 7.4, containing 4 M guanidine HCl, then chilled and precipitated again. The resulting pellet was spiked with [^{3}H]leucine (20000 d.p.m./μl), 0.5 ml of 6 M HCl was added and the samples were capped with poly(tetrafluoroethylene)-faced 8 mm silicone septa and hydrolysed overnight at 110 °C. An aliquot of the hydrolysate was spotted on 2.54 cm × 40 cm Whatman 3MM chromatography paper strips and, after descending chromatography in a 95% (v/v) ethanol/15 M NH_4OH (19:1, v/v) solvent for 22 h, the strip was air-dried and cut into 1.3 cm segments that were counted in minivials with 4 ml of Betafluor scintillation fluid (National Diagnostics, Atlanta, GA, U.S.A.). The radioactivity (c.p.m.) in the separated ^{3}H-labelled peaks were summed to obtain the relative amounts of [^{3}H]proline, [^{3}H]hydroxyproline and [^{3}H]leucine in the sample. The amount of [^{3}H]leucine was used to back-calculate total levels of [^{3}H]hydroxyproline in the original sample (by comparing yield in the chromatography step with the original amount spiked in the sample before hydrolysis).

Transfection studies

A consensus oestrogen-responsive element (ERE) CAT construct, pA2(−482/−87)tk–CAT, and a construct lacking the ERE, pBL–CAT2, were used in transient transfection studies

[26]. Proliferating cell strains from donors of different age were transfected with ERE–CAT or pBL–CAT2 with the calcium phosphate method; the efficiency of transfection was determined by β-galactosidase assay on transfected and control cultures [27]. In brief, cells were incubated with 50 μg of purified DNA (10 μg of pCMVβgal and 40 μg of CAT vector) with 0.25 M $CaCl_2$ in Bes-buffered saline for 18 h at 37 °C in a decreased (2 %) CO_2 environment. After transfection the strains were treated with 0.1 nM oestradiol-17β and cell number, total protein, β-galactosidase activity and CAT activity were determined 24 h later [28]. CAT activity (^{3}H c.p.m. of acetate transferred) was determined by partition-phase liquid-scintillation counting [28]. Activity was measured in ERE–CAT-transfected strains in the presence or absence of oestradiol-17β and in strains transfected with CAT alone in the presence or absence of oestradiol-17β. Triplicate samples were averaged and the values normalized on a 10^6 cell basis. Results are expressed as (^{3}H c.p.m. with oestradiol)/(^{3}H c.p.m. without oestradiol) for comparison.

ERα assay

Osteoblasts were analysed for ERα levels on passage from explant into primary culture. Fibroblast or osteoblast-like cells were seeded at 50000 cells/cm^2. After 3 days of culture, cell strains were rinsed twice with PBS and treated with trypsin to generate a single-cell suspension. An aliquot was counted for cell number and the cells were pelleted. The pellet was resuspended in 3 ml of PBS and freeze–thawed twice; it was then sonicated and the sample was divided in half for duplicate analysis. No diluent was added to the cell strain samples and the 400 mM KCl nuclei extraction was subsequently followed as described [29]. Osteoblast ERα could be measured in 2×10^5–8×10^5 cells, whereas at least 2×10^6 fibroblasts were needed for detection at the low end of the linear region of the assay. Biopsies were analysed for ERα content by following the ERα EIA manufacturer's protocol exactly (Abbott Laboratories). On the basis of data pooled from all kits run in our laboratory so far and values determined for the same samples and controls analysed repetitively, the intra-assay coefficient of variance (CV) was 10 %; the inter-assay CV was 10 %.

Enucleation

Cell strains were treated with or without 0.1 nM oestradiol-17β; after 16 h the cells were enucleated by treatment with cytochalasin B, and the levels of the ERα in the enriched nucleoplast pool and in cohort wells extracted for total ERα were compared to determine cytoplasmic and nuclear localizations of the receptor. Cells were enucleated by the cytochalasin B Percoll step-gradient equilibrium centrifugation method of Welshon et al. [30]. The supernatant layers were combined and taken as the cytoplasmic pool; the lowest layer of Percoll and nucleoplasts were combined and taken as the nuclei pool.

Statistical analysis

Significant differences in collagen synthesis and ERE–CAT activity in response to oestradiol as a function of donor age were determined by regression analysis. The conservative Welch alternative t test, which does not assume equal variances, was used to compare the means of ERα levels between groups of different ages and to determine P values [31]. To follow donor-to-donor variability in ERα levels, the CV was calculated on samples that had two donors or more of the same age. Both linear and logistic regression analyses were performed on ERα levels and donor ages. A physiologically relevant version of the modified logistic equation [32] was derived:

$$\text{ER}\alpha \text{ level} = (a-d)/[1+(A/c)^b]+d$$

where a is pre-menopausal ERα level, d is post-menopausal ERα level, c is the average age midpoint between the two groups (point of inflection), $b = 100$, and A is age in years. Curve-fitting employed the Marquardt–Levenberg algorithm to solve for the coefficients that gave the best fit betwen the equation and results [33].

RESULTS

Osteoblast-like cells derived from three healthy female donors were cultured under well-defined conditions [22] and treated with various concentrations of oestradiol-17β in the presence of [^{3}H]proline. When steady-state collagen synthesis was analysed as [^{3}H]hydroxyproline levels normalized by cell number, a biphasic response was evident in osteoblast-like cells derived from young donors (Figure 1a). In these cell strains an optimal concentration of 0.1 nM oestradiol doubled the levels of collagen. Osteoblast-like cells derived from a 10-year-old donor exhibited a greater response than those derived from a 5-year-old donor. In contrast, the cell strain derived from a 68-year-old donor exhibited no significant response. A similar biphasic response to oestradiol was observed when the levels of ERα were determined by EIA 18 h after treatment with various concentrations of oestradiol (Figure 1b). Again, osteoblast-like cells derived from young female donors (ages 5 and 10) exhibited a biphasic

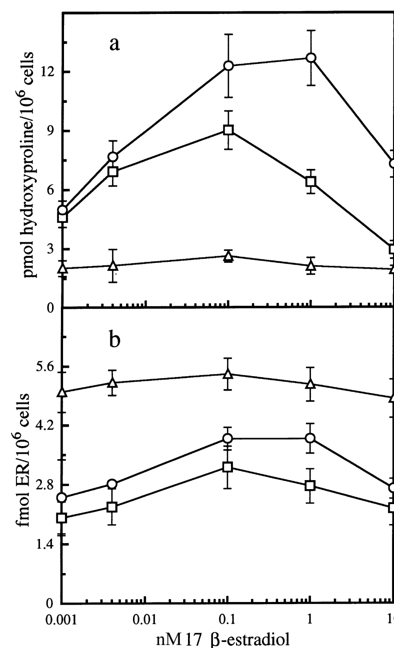

Figure 1 Dose response of osteoblast-like cells to oestradiol-17β

Human osteoblast-like cells derived from three different female donors (□, 5 years old; ○, 10 years old; △, 68 years old) were passaged from explant culture at 40000 cells/cm^2, grown in primary culture for 54 h and treated for 18 h with [^{3}H]proline and various concentrations of oestradiol-17β. At the end of treatment, triplicate wells were harvested for [^{3}H]hydroxyproline determination and triplicate cohort wells were analysed for cell number by trypsin treatment and a Coulter counter. The levels of collagen were determined as pmol of [^{3}H]hydroxyproline per 10^6 cells (**a**). An additional set of triplicate wells were used for ERα analysis with a commercially available enzyme immunoassay and values determined were normalized to cell number (**b**).

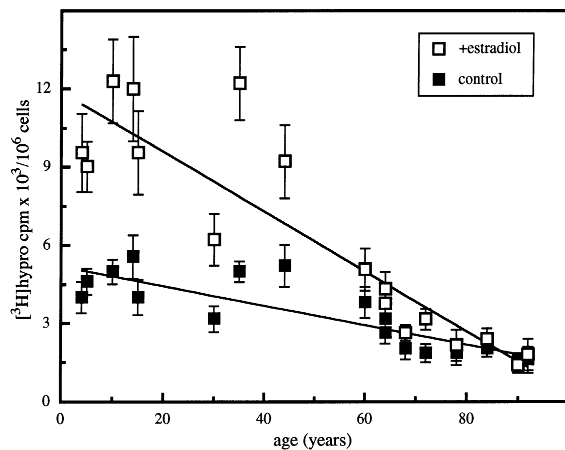

Figure 2 Effect of donor age on stimulation of collagen synthesis by oestradiol-17β

Human osteoblasts from 12 different female donors were radiolabelled with [^{3}H]proline in triplicate in the presence of 0.1 nM oestradiol-17β or vehicle alone. After 18 h the medium and cell layer pools were harvested and the levels of [^{3}H]hydroxyproline derived from the labelled proteins were determined for each osteoblast strain and normalized on a 10^6 cell basis. The equation and statistics for regression analysis of basal collagen levels (control, vehicle only) as a function of donor age was $y = -3.57 \times 10^{-2}x + 5.07$, $r^2 = 0.64$, $P \leqslant 0.01$; the statistics for oestrogen responsiveness (+estradiol) in terms of collagen levels as a function of donor age yielded $y = -1.14 \times 10^{-1}x + 11.4$, $r^2 = 0.85$, $P \leqslant 0.001$.

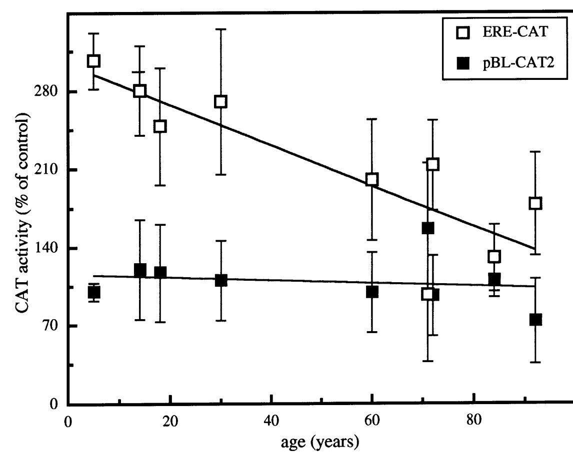

Figure 3 Effect of donor age on ERE activity

Proliferating cell strains from different donors were transfected with 10 μg of pCMVβ and 40 μg of ERE–CAT or pBL–CAT2 construct for 18 h at 37 °C with 2% CO_2 by using the calcium phosphate method, and the efficiency of transfection was determined by β-galactosidase assay on transfected and control samples. CAT activity was measured by partition-phase liquid-scintillation counting 48 h after transfection in ERE–CAT-transfected (□) and CAT-alone-transfected (■) strains with or without oestradiol-17β for 24 h. Triplicate samples were averaged and the values were normalized on a 10^6 cell basis. Results are expressed as (^{3}H c.p.m. with oestradiol)/(^{3}H c.p.m. without oestradiol) for comparison. The variability for each individual cell line is given as an error bar representing the S.D. (assays were done in triplicate). Regression analysis of CAT activity (percentage of control) in ERE–CAT-transfected cells yielded the equation $y = -2.38 \times 10^{-1}x + 3.07 \times 10^2$, $r^2 = 0.66$, $P \leqslant 0.05$.

increase in ERα levels, with an optimal concentration of approx. 0.1 nM, whereas cells derived from the older donor exhibited a minimal response. Suprisingly, the levels of ERα were higher at all doses of oestradiol in the cell strain derived from the 68-year-old donor.

To investigate further the effect of age on oestrogen responsiveness, we obtained bone from 17 healthy women of various ages. Osteoblast-like cells were treated with 0.1 nM oestradiol-17β and radiolabelled to steady state with [^{3}H]proline. The levels of collagen synthesized were determined as [^{3}H]hydroxyproline produced by the different cell strains. Oestrogen treatment caused a more than 2-fold increase in collagen protein levels in strains derived from donors less than 50 years of age (10.00 ± 0.74 compared with 4.56 ± 0.28 pmol of hydroxyproline per 10^6 cells; mean ± S.E.M., $n = 8$ donors). The increase in hydroxyproline levels in osteoblast strains derived from women at least 50 years of age and treated with oestrogen was not significant (2.97 ± 0.41 compared with 2.28 ± 0.25 pmol of hydroxyproline per 10^6 cells; mean ± S.E.M., $n = 9$ donors). An alternative statistical method to analysing data by segregating osteoblast-like cell strains into different donor age groups and comparing mean values was to analyse the data by linear regression. When the effect of donor age on basal collagen protein levels was analysed by regression analysis (Figure 2), a significant negative correlation was seen ($r^2 = 0.65$, $P \leqslant 0.01$). Regression analysis of donor age and oestradiol-stimulated collagen synthesis also yielded a significant negative correlation ($r^2 = 0.80$, $P \leqslant 0.0001$). Furthermore the rate of age-related decrease in oestradiol responsiveness (slope of the regression line) was significantly different from the basal age-related decrease in collagen synthesis ($P \leqslant 0.01$). Thus regression analysis showed a significant diminution in oestradiol responsiveness with increasing donor age.

Next the functionality of the ER in terms of receptor inducibility and activity was assessed. Osteoblast-like strains derived from nine healthy female donors of different ages were transiently transfected with a pBL–CAT2 or an ERE–CAT vector. The latter construct contains a consensus ERE and the promoter for tk in place of the oestrogen promoter. The transfected strains were treated with 0.1 nM oestradiol-17β in serum-free, Phenol Red-free defined DMEM medium and the percentage transfection (by assaying β-galactosidase activity), CAT activity, total protein and cell number were determined after 24 h. The CAT activity of the two constructs was expressed as a percentage of control (no oestradiol added). Treatment with oestradiol caused CAT activity in the ERE–CAT-containing strains derived from donors less than 50 years of age to increase approx. 2-fold (276 ± 12 compared with 112 ± 4; mean ± S.E.M., $n = 4$), whereas strains derived from donors at least 50 years of age exhibited lower stimulation of CAT activity (164 ± 18 compared with 107 ± 14; means ± S.E.M., $n = 5$, where 1 unit of CAT activity is defined as c.p.m. chloramphenicol acetylated/2 h per μg of protein under standard conditions). In strains containing pBL–CAT2 and treated with oestradiol, CAT activity was approx. 100% of control (no oestradiol treatment) at almost all ages. Regression analysis (Figure 3) yielded a significant negative correlation between ERE–CAT activity in response to oestradiol as a function of donor age ($r^2 = 0.66$, $P \leqslant 0.05$). Although basal CAT activity in the absence of an ERE yielded a reasonable regression coefficient ($r^2 = 0.63$), the slope was not statistically significantly different from zero (i.e. basal CAT activity was constant with donor age).

To determine whether the age-related decrease in responsiveness was correlated with changes in ER number, osteoblasts derived from 52 healthy women were cultured in normal serum and Phenol Red-containing medium and their ERα levels were determined 72 h after passage to primary culture by using an

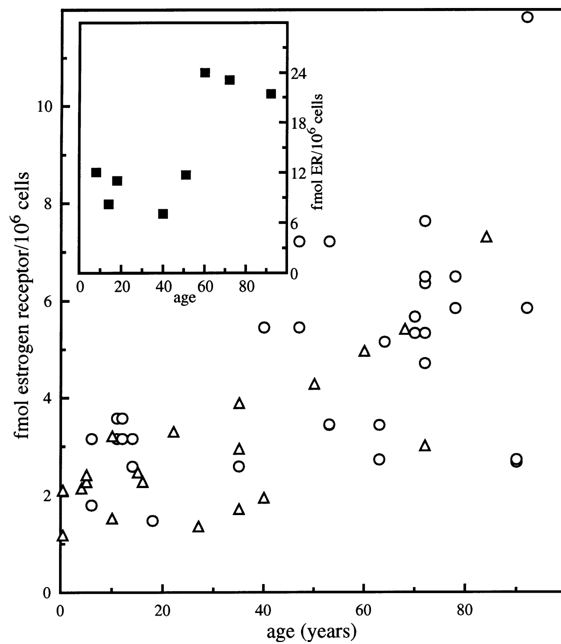

Figure 4 ERα contents in osteoblast strains derived from donors of different ages

Human osteoblasts strains from surgical specimens (○) or cadaveric donations (△) were seeded at 50000 cells/cm^2 and the ERα content was determined by EIA after 72 h of culture. Inset: normal female human osteoblasts were grown in primary culture in serum-free, Phenol Red-free defined medium, passaged at 50000 cells/cm^2 into serum-free, Phenol Red-free defined medium and after 72 h of culture the levels of ERα were determined by EIA.

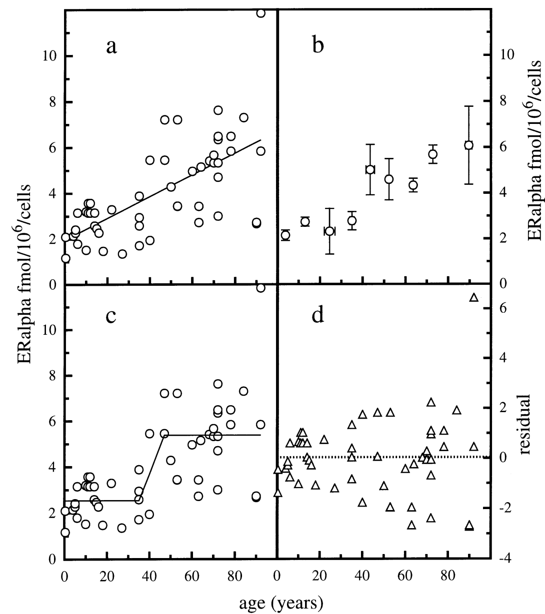

Figure 5 Modelling of aging and ERα levels

ERα levels in strains of osteoblast-like cells derived from 52 different donors were plotted as a function of donor age and subjected to linear regression, yielding $y = 4.68 \times 10^{-2}x + 1.44$ (**a**). ERα levels in strains derived from 52 different donors were stratified by donor age into decade groups and the means and standard error were plotted for ERα and age group (**b**). ERα and donor age were analysed by logistic regression by fitting the ERα values determined to a modified logistic equation as a function of donor age (**c**). Residual values were calculated on the basis of the logistic equation and plotted as a function of donor age (**d**).

EIA that measured total (free plus liganded) receptor present in both cytoplasm and nucleus [29]. The results were normalized on a 10^6 cell basis and revealed a distinct grouping of ERα levels as a function of age (Figure 4). As an initial approach to analysis, cell strains were stratified by donor age range (group I, less than 40 years of age; group II, 40–55 years of age; group III, more than 55 years of age, approximating pre-menopausal, peri-menopausal and post-menopausal age groups) and means ± S.E.M. of concentration (fmol per 10^6 cells) were compared. The mean for group I was 2.54 ± 0.16 fmol per 10^6 cells ($n = 24$, mean age 15, age range 0–35 years), that for group II was 4.80 ± 0.66 fmol per 10^6 cells ($n = 8$, mean age 48, age range 40–53 years), and that for group III was 5.44 ± 0.47 fmol per 10^6 cells ($n = 20$, mean age 75, age range 60–92 years). The conservative Welch alternative t test, which does not assume equal variances, was used to compare mean values for group I and III and yielded $P \leqslant 0.0001$.

Because 17 of the 28 different donor ages included in the study had two donors (or more) of the same age, and analyses were done in duplicate, the values determined for each age were combined and averaged, and the CV was calculated for each age group. The average CV for all groups combined was 0.25. The CV values for groups I, II and III were 0.25, 0.44 and 0.18 respectively. That the largest variance was found in the group of donors between 40 and 55 years of age is consistent with the transitional nature of this peri-menopausal group. When fmol per 10^6 cell values were converted to averages ± S.E.M. in terms of molecules per cell, we estimated there to be 1500 ± 100 molecules per cell for the cell strains derived from younger women (group I) and 3300 ± 300 molecules per cell for cell strains derived from older women (group III). These values are similar to those reported for human osteoblast-like cells derived from normal women (age range undefined) that were determined by [^{3}H]oestradiol nuclear binding assay, in which approximately 2000 binding sites per cell were found [7].

In determining ERα levels, we initially analysed various osteoblast-like strains that were cultured in DMEM medium containing 10% (v/v) FBS and Phenol Red. The concentration of oestrogen in this medium, as determined by EIA and analysis of ten different lots of serum and medium, was found to be 4.1 ± 1 pM. Normal mean mid-cycle serum levels of oestradiol were 551 pM for adult women less than 40 years old and 47.7 pM for women more than 55 years old. Although oestrogen was present during the culture of these cells, on the basis of the observed differences in receptor number, it seems that 4 pM oestradiol was not sufficient to alter these relative age-related differences. When osteoblast-like cell strains derived from eight female donors of different ages were cultured under serum-free, Phenol Red-free defined medium, the levels of ERα per 10^6 cells were increased almost 4-fold (Figure 4, inset). Suprisingly, the age-related difference in receptor number was maintained under serum-free, Phenol Red-free conditions, with strains derived from older donors having over 2-fold higher levels than in strains derived from younger women (means ± S.E.M. were: group I, 10.40 ± 1.14, $n = 3$; group II, 9.38 ± 2.33, $n = 2$; group III, 22.83 ± 0.76, $n = 3$). Comparison of the means of groups I and III by Welch's alternative t test yielded $P \leqslant 0.01$.

A second approach to analysing ERα levels statistically as a function of donor age was to do a linear regression analysis. The results of this analysis (Figure 5a) yielded regression parameters of $r^2 = 0.42$ and $P \leqslant 0.001$. Thus the slope is significantly different from zero and linear regression accounts for 42% of the scatter

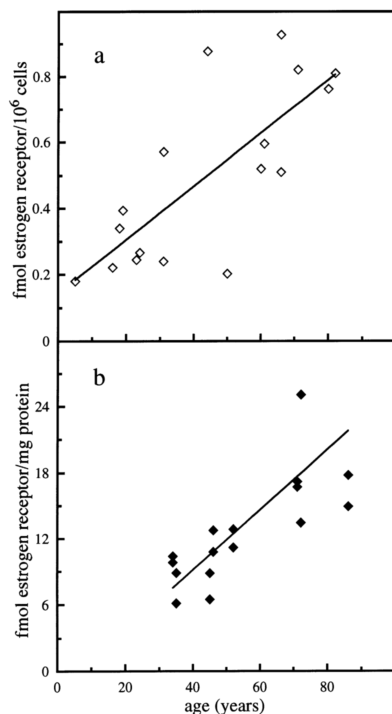

Figure 6 ER content in normal female fibroblast and skin

Normal human fibroblasts were seeded at 50 000 cells/cm^2 into Earle's Minimal Essential Medium containing Earle's salts and 10% (v/v) FBS and cultured for 3 days before harvesting and analysis for ER levels by EIA (**a**). ER content in dermal biopsies from normal women on no hormonal therapy was determined by ER EIA (**b**). Before analysis the epidermis and fatty distal portion of the dermis were removed surgically from the frozen sample and the biopsy was halved.

of the data. The difficulty in applying linear regression analysis to any given data set lies in whether the mathematical model is physiologically relevant. That is, the use of a linear model of ERα levels as a function of human osteoblast-like cell donor age implies a one-to-one correspondence between ERα and age throughout the entire age range. Thus ERα levels would increase from birth onward at a constant rate (slope). When ERα levels were grouped by donor age by decades, a non-linear pattern was observed in which two distinct populations were linked by a transition phase between the fourth and fifth decade (Figure 5b). A well-established mathematical model for fitting such data is a logistic regression, in which two 'steady-state' populations are linked by a phase of rapid transition. Receptor levels were modelled by fitting a modified logistic equation to ERα and donor age values and determining the values of the coefficients a, d and c (Figure 5c). The resultant value for the average 'pre-menopausal' ERα level (coefficient a) was 2.5 ± 0.3 fmol per 10^6 cells, that for the average post-menopausal ERα level (coefficient d) was 5.4 ± 0.3 fmol per 10^6 cells, and the point of inflection (coefficient c) was 42 years. A regression coefficient of 0.71 was obtained; thus logistic regression accounted for 50% of the data's scatter. A plot of residual values yielded a random distribution above and below zero with no sequential positive or negative runs, indicating a respectable fit to the data (Figure 5d). The Durbin–Watson statistic (a measure of correlation between the residuals) was 2.06, indicating that the residuals were independent of each other (as assumed by regression analysis). The assumption that the source population was normally distributed about the logistic regression line was found to be valid with the Kolmogorov–Smirnov test.

The ERα EIA that we employed measures total (cytoplasmic plus nuclear) receptor levels, and it was possible that a change in receptor partitioning might have given rise to an altered response to oestradiol-17β. Therefore it was of interest to compare nuclear and cytoplasmic ERα levels as a function of osteoblast donor age. Six osteoblast-like strains (derived from donors aged 14, 18, 30, 60, 72 and 92 years) were grown in serum-free, Phenol Red-free defined medium and the distribution of the receptor was determined after cytochalasin B-induced enucleation. No age-related differences in cytoplasmic or nuclear residence of the receptor were observed between the six different strains. Samples grouped as derived from donors less than 50 years old had an average ERα percentage nuclear localization of 64 ± 7.6%, whereas strains derived from donors more than 50 years of age had an average ERα nuclear localization of 52 ± 10%.

To determine whether the age-related changes in ERα were unique to bone cells, the levels of the receptor in human fibroblasts derived from 17 different female donors were determined under standardized culture systems. The results (Figure 6a) indicated that the ERα levels per 10^6 cells increased with increasing age. ERα levels were more than 2-fold higher in the fibroblasts derived from older donors. However, fibroblast ERα levels were one-tenth those in osteoblasts. We next looked for correlation *in vivo* of our data *in vitro*. Dermal biopsies from healthy women not on hormone replacement therapy were analysed directly for ERα content. Normalizing ERα levels to cytosolic protein content showed an age-related increase in ERα concentration (Figure 6b). Again, a more than two-fold increase in ERα levels was observed between biopsies derived from older and from younger donors. Linear regression analysis of ERα levels as a function of donor age in normal human female fibroblasts yielded an r^2 of 0.58 and $P \leqslant 0.001$. A similar analysis of ERα levels in normal human dermal biopsies yielded linear regression parameters of $r^2 = 0.59$ and $P \leqslant 0.0005$. Analysis by logistic regression was not feasible in view of the relatively small number of donors in each group.

DISCUSSION

The hypothesis that aging is associated with alterations in the levels or function of ER in bone has not been well studied. However, such studies have been done in mouse [34–38] and rat brain and uteri [39–46] where, in general, decreased ER binding was observed with age. These studies employed assays in which receptor levels were determined by radioactive oestradiol binding in nuclei. By this methodology, receptors with altered ligand-binding activity might not be detected. It is possible that altered receptor affinity might arise from differential splicing of ER transcripts resulting in modified receptor affinity or activity with aging [47]. The contrasting results between studies in rats and mice and our current study in humans could be a reflection of the different assays used. The oestradiol nuclear binding assay measures functional binding ability (ligand affinity), whereas an EIA measures total receptor levels. The possibility that ligand-binding assays might yield different results than antibody-based assays was first proposed by Roth in 1975 [48].

Recently a second ER gene (ERβ) has been identified in rat, mouse and humans [8–10,12]. In humans ERβ expression has been localized to thymus, spleen, ovary, testis [10] and osteoblasts [49]. ERβ can form functional heterodimers with ERα that might modulate ERα activity [50–52]. Evidence suggesting that ERβ acts in a negative fashion relative to ERα in regulating AP1 site transcription has recently been reported [53]. The monoclonal

antibody used in the ERα EIA (H222) has no detectable cross-reactivity with ERβ produced by the osteoprogenitor human ERα knock-out (HERKO) cells (N. Fedarko and M. Young, unpublished work). This cell line was derived from a 28-year-old male donor with a mutation in both alleles of the ERα gene [54,55]. Thus our observation of age-related changes in receptor levels are specific for ERα. However, it is possible that the age-related diminution of osteoblast-like cell responsiveness to oestrogen might involve modulation by ERβ of the normal ERα repsonse.

Unlike membrane-associated growth factor receptors, which exhibit ligand-dependent up-regulation and down-regulation, ER regulation by its cognate ligand seems to be organ-dependent. Treatment of rats with oestrogen caused ER levels in the testes to decrease [56], whereas ER levels in the uterus and vagina increased [57]. The results of the current study suggest that ER levels in osteoblast-like cells are up-regulated by age-dependent decreases in oestrogen. Furthermore when bone cells were cultured in medium lacking Phenol Red or any detectable oestrogen, induction of receptor was observed independently of donor age. This effect has been seen both in cells derived from human tissue (bone and skin) and in intact dermal biopsies, implying that this was not an artifact *in vitro*. That fibroblasts behave similarly to osteoblast-like cells was not suprising, given that skin exhibited changes in collagen levels and mechanical properties with menopause, and oestrogen replacement therapy reversed these changes [21,58]. Furthermore skin collagen levels and bone mineral density were highly correlated with each other during aging [59].

It should be noted that 20 of the 52 osteoblast-like strains analysed for ERα levels were obtained from trauma victims. Of the remaining 32 strains, 20 were obtained from iliac crest bone intended for bone graft and 12 were from uninvolved distal portions of the femoral neck removed during hip arthroplasty. Therefore, even though it is possible that the pathological condition causative of hip arthroplasty (degenerative joint disease) might give rise to abnormal results in assays *in vitro*, this could only effect one-quarter of the osteoblast-like cell strains analysed. Additionally it should be noted that there were no statistically significant differences in ERα levels between age-grouped cell strains obtained from trauma victims, for bone graft or during hip surgery.

In osteoblast-like cells, the magnitude of an end result of oestrogen binding to ERα (collagen synthesis) and the ability of oestrogen to induce CAT activity via ERα–ERE–CAT interactions were decreased with increasing age. These results parallel the generalized decrease in adrenergic responsivity with aging observed in multiple cells and tissue types [48,60–66]. The decrease in adrenergic response was associated with increasing adrenergic receptor density and dysfunction of the receptor signal-transducing pathway [66,67]. Age-associated diminution of signal transduction might therefore be a likely mechanism to explain our observations. Within this context, the increase in ER levels with age might be a consequence of decreased ER responsivity reflecting a compensatory mechanism of the cell.

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