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Phytoestrogen Genistein Acts as an Estrogen Agonist on Human Osteoblastic Cells Through Estrogen Receptors α and β

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Abstract Genistein, a soybean isoflavone, has estrogen-like activity in mammals, including the prevention of bone loss. However, whether its mechanism of action on bone turnover is distinct from that of estrogen or raloxifene is unknown. Although genistein has been reported to bind both estrogen receptor (ER) isoforms (α and β), little is known concerning differential activation of gene expression via these ER isoforms. To examine this question, comparison of the responses of normal fetal osteoblast (hFOB) cells stably expressing either ERα (hFOB/ERα9) or ERβ (hFOB/ERβ6), to treatment with genistein, 17β-estradiol (E_2) or raloxifene were conducted. In hFOB/ERα9 cells, both genistein and E_2 increased the endogenous gene expression of the progesterone receptor (PR), the proteoglycan versican, and alkaline phosphatase (*AP*), but inhibited osteopontin (*OP*) gene expression and interleukin-6 (IL-6) protein levels. Raloxifene had no effect on these bone markers. Genistein, but not raloxifene, also mimicked E_2 action in the hFOB/ERβ6 cells increasing PR gene expression and inhibiting IL-6 production. To determine whether the gene regulatory actions of genistein in human osteoblast cells occur at the level of transcription, its action on the transcriptional activity of a PR-A promoter-reporter construct was assessed. Both genistein and E_2 were found to stimulate the PR promoter in the hFOB cell line when transiently co-transfected with either ERα or ERβ. Whereas hFOB cell proliferation was unaffected by E_2, raloxifene or genistein at low concentrations, higher concentrations of genistein, displayed significant inhibition. Together, these findings demonstrate that genistein behaves as a weak E_2 agonist in osteoblasts and can utilize both ERα and ERβ. J. Cell. Biochem. 89: 633–646, 2003.

Key words: phytoestrogens; genistein; estrogen receptor; osteoblasts; progesterone receptor; IL-6; versican; alkaline phosphatase; osteoporosis

Phytoestrogens are a structurally diverse group of plant-derived compounds that include the isoflavones, lignans, and coumestans. Due to their estrogen-like activity in mammals, there is considerable interest in phytoestrogens as potential natural alternatives or supplements to traditional hormone replacement therapies for postmenopausal osteoporosis, cardiovascular disease, as well as the treatment of reproductive cancers [Glazier and Bowman, 2001]. The lower incidence of osteoporosis in Japan and of hip fractures in Asian populations in general, compared to Caucasians, may be correlated with the higher consumption of foods rich in phytoestrogens [Cooper and Campion, 1992; Russell-Aulet et al., 1993]. However, a causal link between phytoestrogens and bone mass maintenance in humans has yet to be established. Nevertheless, short-term treatment of peri- and post-menopausal women with soy protein diets enriched in isoflavones has been demonstrated to reduce bone loss from the lumbar spine as well as provide a potential cardioprotective effect through improvement of the serum cholesterol profile [Potter et al., 1998; Alekel et al., 2000]. Long-term (1–2 year) treatment with the synthetic isoflavone, ipriflavone, not only potentiated the effect of low dose estrogen in reducing bone loss in healthy postmenopausal women [Agnusdei et al., 1995],

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but also prevented the loss of cortical and cancellous bone when administered alone to osteopenic postmenopausal women [Passeri et al., 1992; Gennari et al., 1997]. Soybean protein and natural and synthetic isoflavones have also been demonstrated to prevent sex-steroid deficiency-induced bone loss and elevated blood cholesterol in ovariectomized rodents [Arjmandi et al., 1998; Uesugi et al., 2001]. However, as with estrogen, these compounds cannot reverse established osteopenia [Arjmandi et al., 1998; Picherit et al., 2001].

The mechanism of action of isoflavones in the prevention of bone loss appears to differ from that of estrogen and selective estrogen receptor modulators (SERMs, i.e., raloxifene). Whereas both estrogen and raloxifene prevent bone loss by reducing bone resorption [Wronski et al., 1988; Evans et al., 1996], the isoflavones genistein and ipriflavone, are antiresorptive only at chronic high doses and therefore have been reported to either inhibit [Gennari et al., 1997; Picherit et al., 2001] or not affect [Passeri et al., 1992; Agnusdei et al., 1995; Uesugi et al., 2001] the bone turnover rate. Indeed, high dose (>1 μM) ipriflavone and ipriflavone metabolites have been reported to inhibit multinucleated osteoclast formation and activity in vitro [Notoya et al., 1993; Giossi et al., 1996]. Interestingly, neither of these isoflavones blocks the elevated bone resorption rates induced by ovariectomy or menopause [Gennari et al., 1997; Arjmandi et al., 2000]. Thus, while isoflavone treatment may prevent bone loss, in part, because the enhanced bone formation exceeds resorption, the mechanism(s) underlying the enhanced bone formation by isoflavone treatment in vivo is largely unknown. In immature and mature osteoblast cell cultures certain isoflavones, including genistein, have been shown to modestly stimulate differentiated osteoblast characteristics, such as the synthesis of total proteins [Yamaguchi and Sugimoto, 2000], type I collagen, alkaline phosphatase (AP) activity [Benvenuti et al., 1991], and mineralized matrix deposition [Cheng et al., 1994].

Mechanistically, genistein is considered to be one of the most estrogenic of the phytoestrogens, and is able to bind both α and β isoforms of the estrogen receptor (ER). Both osteoblasts and osteoclasts express ERs and respond to estrogen treatment in vivo and in vitro [for a review see Oursler, 1998]. Although the affinity of genistein for ERα and ERβ is lower than that of 17β-estradiol (E_2), the relative binding affinity of genistein for ERβ is greater than for ERα [Kuiper et al., 1998]. The two ER isoforms exhibit differential ligand-dependent regulation not only of transcription from synthetic inducible promoter elements [Paech et al., 1997; Saville et al., 2000] but also of endogenous genes and cellular functions in osteoblasts in vitro [Waters et al., 2001]. Thus, the repertoire of target genes affected, as well as the response of any specific gene, will be dependent on the particular ligand bound to the receptor isoforms and the ratio of ERα to ERβ. Thus, the divergent effects of isoflavones on bone metabolism in vivo, including their unique regulation of osteoblast and osteoclast functions, could be caused by the relative concentrations of the ERα and ERβ isoforms in the osteoblasts and other skeletal cells. To examine this possibility, the effects of genistein on human osteoblast cell lines, stably expressing either the ERα or ERβ isoform, were compared to the effects of E_2 and the SERM raloxifene.

MATERIALS AND METHODS

Reagents

The phenol red-free Dulbecco's MEM-Ham's F12 (1:1) medium (DMEM-F12), DMEM medium, and all other reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Charcoal-stripped fetal bovine serum (CS-FBS) was purchased from Hyclone Laboratories (Logan, UT), hygromycin B from Boehringer Mannheim (Indianapolis, IN), zeocin from Invitrogen (Carlsbad, MA), geneticin disulphate (G418) and Lipofectamine PLUS transfection reagent from Gibco BRL (Gaithersburg, MD). Human recombinant basic fibroblast growth factor (bFGF) was purchased from Promega Corporation (Madison, WI), [^{3}H] methyl-thymidine from DuPont-New England Nuclear (Boston, MA), and α-[^{32}P]-dCTP from Amersham Pharmacia (Piscataway, NJ).

ICI 182,780 was generously provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Plasmid reporter constructs containing the human PR_A promoter region was generously provided by Prof. Pierre Chambon (University Louis Pasteur, INSERM, Strasbourg, France) [Kastner et al., 1990]. Full-length human versican cDNA and crude rabbit anti-human versican polyclonal antiserum

were a generous gift from Dr. Erkki Ruoslahti (The Burnham Institute, La Jolla, CA) [LeBaron et al., 1992]. The human AP cDNA probe was a gift from Dr. Gideon Rodan (Merck, Sharp and Dohme, West Point, PA), and the osteonectin cDNA probe was provided by Dr. George Long (University of Vermont, Burlington, VT).

Purification of Raloxifene

Raloxifene (LY 139,481) was purified from Evista® tablets (Eli Lilly, Indianapolis, IN). Each tablet containing 60 mg raloxifene HCl (MW 510.05) was pulverized and then extracted in absolute ethanol for 1 h at 4°C. Insoluble material was removed by filtration and the supernatant dried under vacuum. The dried residue was dissolved in 10% (v/v) acetonitrile in water, 0.1% (v/v) trifluoroacetic acid (TFA), and fractionated on a C-18 HPLC column using a 4% to 64% (v/v) acetonitrile/water gradient containing 0.1% (v/v) TFA with detection at 220 nm. A single peak eluting around 40% acetonitrile was collected and pooled fractions desiccated, resuspended in methanol/water and then re-desiccated. The resultant pale yellow powder was then analyzed by mass spectrometry and produced a single spike with MW 474.1, the expected molecular mass of raloxifene. Purified raloxifene was then dissolved in absolute ethanol to yield a 1 mM stock solution. The biological activity of the purified raloxifene was verified by testing its antagonism of E_2-stimulated MCF-7 breast cancer cell proliferation (Fig. 1) essentially as described [Wakeling et al., 1984].

Cell Culture

The hFOB/ERα9 and hFOB/ERβ6 human fetal osteoblast cell lines were developed from the parent temperature-sensitive SV40 (tsA58) large T antigen-immortalized hFOB1.19 cell line by stable transfection with ERα and ERβ expression plasmids, respectively, and have been described previously [Harris et al., 1995; Waters et al., 2001]. These cells express only the single ER isoform, as appropriate, and exhibit E_2 responsiveness. Cells were cultured in phenol red-free DMEM-F12 medium supplemented with 10% (v/v) CS-FBS with continual selection in alternating antibiotics [Waters et al., 2001]. The hFOB1.19 cells were cultured in DMEM-F12 supplemented with 10% (v/v) FBS and G418. All experiments were carried out at the permissive temperature of 34°C.

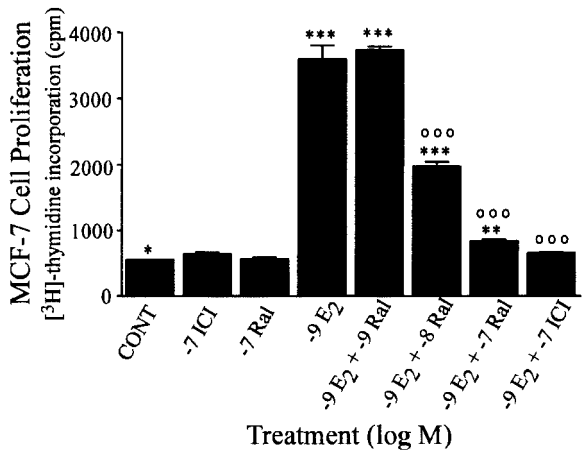

Fig. 1. The HPLC purified raloxifene antagonism of the E_2 induction of MCF-7 cell proliferation. MCF-7 cells were pretreated with ICI and treated with E_2, raloxifene (Ral), or ICI in DMEM-F12 + 1% (v/v) CS-FBS. DNA synthesis was measured after 3 days treatment by the incorporation of [^{3}H]-thymidine. The data are presented as the mean ± SEM of six determinations for each treatment. A representative experiment repeated four times with similar results is shown. Significant differences between the control (i.e., treatment with ICI alone) and the other treatments are indicated by asterisks (*) while the significant differences between treatment with E_2 alone and estrogen co-treatments with either raloxifene or ICI are denoted by $^{000}P < 0.001$.

MCF-7 human breast carcinoma cells were cultured in phenol red-free DMEM-F12 medium containing 10% (v/v) FBS and MG63 human osteosarcoma cells in DMEM medium supplemented with 10% (v/v) FBS, both at 37°C.

Cell Proliferation

Cells were seeded into 12-well dishes (4 cm^2/well) at a density of 2×10^4 (hFOB) or 2×10^5 (MCF-7) cells per well, and allowed to recover for 24 h. Cells were then pretreated for 48 h with ICI 182,780 (10^{-8} M) in DMEM-F12 + 10% (v/v) CS-FBS (5% CS-FBS for MCF-7 cells) without antibiotics. The ICI 182,780 treatment serves to decrease any estrogen-independent signaling (basal signaling). After rinsing three times with serum-free medium, cells were treated with ICI, E_2, raloxifene, or genistein in the presence and absence of bFGF, in DMEM-F12 + 1% (v/v) CS-FBS and cultured either for 3 days (MCF-7 cells) or 5 days (hFOB cells). Cells were labeled with [^{3}H]-thymidine (1 μCi/well) for the final 20 h of treatment. For the hFOB cells, all incubations were at 34°C. As an indicator of DNA synthesis, the radioactivity incorporated into trichloroacetic acid-precipitable material was determined as described [Robinson et al., 1997].

Northern Blotting

Total RNA was extracted from cells using a phenol-guanidine isothiocyanate solution (Tri-Reagent, Molecular Research Center, Cincinnati, OH). RNA, 10 or 15 μg, was separated on 1% (w/v) glyoxal agarose gels and transferred overnight to nylon membranes (Osmonics, Inc., Intermountain Scientific, Bountiful, UT) by downward capillary action in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Membranes were analyzed for steady state mRNA levels by hybridization with cDNA probes labeled by random priming with [α^{32}P]-dCTP using a commercially available kit (DuPont-New England Nuclear). Hybridizations were performed in 50% (v/v) deionized formamide, 3× SSPE (0.45 M NaCl, 30 mM NaH_2PO_4, 3 mM EDTA, pH 7.4), 5× Denhardt's solution (0.25% (w/v) SDS, 10 μg/ml polyadenylic acid), and 0.2 mg/ml denatured salmon sperm DNA. The following cDNA probes were used: progesterone receptor (PR) [Rickard et al., 2002], full length human versican, bone/liver/kidney AP, osteopontin (OP), and osteonectin (ON). Hybridizations and post-hybridization washes were performed at 42°C, except for PR, which was performed at 37°C. Loading and integrity of the RNA was assessed by a final hybridization with a GAPDH cDNA probe. Densitometry of bands on autoradiograms was performed using a DC120 zoom digital camera and 1D image analysis software (Eastman Kodak, Rochester, NY).

Reverse Transcriptase PCR

Aliquots of total RNA (~30 μg) were digested with RNase-free DNase1, and 4 μg of the digested RNA reverse transcribed and amplified in duplicate by PCR using previously described methods [Rickard et al., 1998]. The sensitivity and accuracy of PCR was improved by the inclusion of Taq Start antibody in the reaction mixture to provide a 'hot start' (Clontech, Palo Alto, CA). Amplification reactions were performed for the following sequences: GAPDH; PR hormone binding domain (5′ primer: 5′-AGAGTTGTGAGAGCACTGGAT, and 3′ primer: 5′-GATTCTTTCATCCGCTGTTCAT); and the V0, V1, and V2 splice variants of human versican using published primer sequences [Dours-Zimmermann and Zimmerman, 1994]. The reactions were terminated during the linear amplification phase; after 24 cycles for GAPDH, 38 cycles for PR, and 30 cycles for the versican isoforms. Similar reaction profiles were used for each primer set: an initial denaturation at 94°C for 2 min was followed by cycles of denaturation at 94°C for 30 s, annealing at 55°C for 2 min (50°C for versican V0, and 52°C for versicans V1 and V2), and polymerization at 72°C for 2 min. Amplification products were visualized on 1.5% (w/v) agarose gels stained with ethidium bromide.

Transient Transfection

The hFOB cells seeded into six well plates were transiently transfected at ~70% confluence using the Lipofectamine PLUS reagent (Invitrogen). Each well received 0.5 μg of either ERα or ERβ expression vectors, 2 μg of the reporter construct PR-(464,1105)-CAT containing the human PR_A promoter [Kastner et al., 1990], and 0.5 μg CMV-Luciferase vector to control for transfection efficiency. The ER expression vectors encoded N-terminally FLAG tagged receptor and were constructed in the vector pcDNA4/TO (Invitrogen). After a 3 h incubation, the transfection medium was removed and cells treated overnight with ICI 182,780 (10^{-8} M) in DMEM-F12 + 10% (v/v) CS-FBS. The cells were then rinsed three times with serum-free medium and treated with either E_2 or genistein for 24 h in medium containing 0.1% (w/v) BSA. Each treatment was added to triplicate wells. The CAT and luciferase activities were then assayed in 25 μg and 10 μg aliquots of cell lysate, respectively, as detailed previously [Harris et al., 1995].

Indirect Immunofluorescence for Versican Protein

Versican protein was detected by indirect immunofluoresence of cells stimulated for 48 h. The hFOB/ERα9 cells were seeded at 1.5×10^4 cells/well onto Permanox plastic 8-well chamber slides (Nunc, Inc., Naperville, IL) previously coated with 5 μg/cm^2 human fibronectin. Cells were incubated for 48 h in medium + 10% (v/v) CS-FBS to recover and then stimulated for 48 h with or without E_2, genistein or ICI 182,780 in medium + 0.1%(w/v) BSA. MG63 cells seeded into chamber slides coated with poly-L-lysine (10 μg/cm^2) were used as a positive control [LeBaron et al., 1992]. After fixation and permeabilization with methanol for 15 min at −20°C, cells were rehydrated with PBS + 2% (v/v) FBS, blocked in PBS + 10% (v/v) FBS, and

incubated with rabbit polyclonal anti-human versican antiserum diluted 1:100 in PBS + 3% (w/v) BSA for 1 h at room temperature. Purified rabbit IgG (5 μg/ml) was used as a negative control. The cells were washed in PBS + 0.5% (v/v) Triton X-100, incubated with goat anti-rabbit FITC conjugated secondary antibody diluted 1:200 in PBS + 3%(w/v) BSA, and washed extensively with PBS before being mounted with 90% (v/v) glycerol, 10% (v/v) PBS and 1% (w/v) DABCO. The staining was visualized with an Axioplan 2 fluorescence microscope (Carl Zeiss, Germany).

IL-6 Protein Assay

The hFOB/ER cells were plated into 12 well plates at 4×10^4 cells/cm^2, cultured to confluency and treated for a total of 4 days with either vehicle, ICI 182,780, E_2, raloxifene or genistein at the concentrations indicated in the figure legends, in medium + 10% (v/v) CS-FBS. The medium was changed and fresh treatments added after 2 days. The concentration of IL-6 in conditioned medium harvested on day 4 was measured using an ELISA specific for IL-6, with a sensitivity of 0.7 pg/ml (R&D Systems, Inc., Minneapolis, MN). Each sample was assayed in duplicate with inclusion of a standard curve of recombinant human IL-6 in each assay run.

Statistical Analyses

The results are presented as the mean ± SEM. The non-paired Student's *t*-test was used to calculate statistical significance between the control group and each treatment group. A value of P equal to or below 0.05 was considered to be significant. Statistical significance is denoted in the figures by asterisks: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Verification of the Bioactivity of Purified Raloxifene

Raloxifene, purified from Evista® tablets, exhibited biological activity and behaved as an ER antagonist in MCF-7 breast cancer cells. The proliferation of these cells was induced in a dose-dependent manner by E_2 and the response was inhibited by co-treatment with raloxifene (Fig. 1). A 100-fold molar excess of raloxifene or ICI 182,780 reduced the E_2-stimulated proliferation to basal levels. Raloxifene alone had no effect on the proliferation of MCF-7 cells. In the absence of ICI pretreatment, the proliferative response to E_2 was severely diminished or undetectable, despite the relatively abundant expression of ER in this cell type.

Regulation of PR Gene Expression and Promoter Activity

Expression of the PR gene is stimulated by E_2 in osteoblasts via ERα [Harris et al., 1995; MacNamara and Loughrey, 1998] and to a minor extent by ERβ but only after long-term treatment [Rickard et al., 2002]. To determine whether genistein can also induce PR gene expression in a similar fashion by either ERα and ERβ, the steady state mRNA levels for both PR isoforms were first examined in hFOB/ERα9 (Fig. 2A) and hFOB/ERβ6 (Fig. 2B) cells by Northern blot and RT-PCR analysis, respectively. Genistein (10^{-7} and 10^{-6} M) and E_2 (10^{-10} to 10^{-8} M), but not raloxifene, markedly induce the concentrations of multiple PR transcripts in the hFOB/ERα9 cells after 24-h treatment. The major mRNA species of ~11 kb was increased approximately 10-fold compared to levels in untreated cells. Genistein at 10^{-8} M was non-stimulatory (data not shown), but 100× higher concentrations of genistein than E_2 concentration was shown to stimulate PR mRNA expression by a magnitude similar to that produced by E_2.

As previously reported, the expression of PR mRNA is stimulated in the hFOB/ERβ6 cell by E_2 to considerably less degree and only after lengthy treatment periods compared to the hFOB/ERα9 cells [Rickard et al., 2002]. The PR mRNA in the hFOB/ERβ6 cells was detectable only by RT-PCR following long-term treatment with the steroid for 4 to 14 days. The induction of PR mRNA expression occurred with both genistein (10^{-6} M) and E_2 (10^{-9} to 10^{-8} M) in the hFOB/ERβ6 cell line following treatment for 10 days (Fig. 2B). Genistein at 10^{-6} M was also stimulatory, as was 10^{-7} M genistein (data not shown). Raloxifene at 10^{-7} M failed to induce PR gene expression in the ERβ cells even after 10 days of treatment. Thus, in the hFOB/ERβ6 cells, the effects of genistein on PR mRNA concentrations was weakly stimulatory, but only at approximately 100-fold higher concentrations than E_2. The above results support the notion that genistein is a weaker stimulator of PR gene transcription compared to E_2 when acting through either ER isoform. Further, as reported previously with E_2

[Rickard et al., 2002], the ERα isoform bound by genistein appears to be a more efficient stimulator of PR gene expression than ERβ bound genistein. However, due to the lack of specific, high affinity antibodies to ERβ, the relative abundance of ER protein between the two hFOB/ER cell lines is unknown.

To better assess the actions of genistein under conditions of comparable ERα and ERβ receptor levels, and to determine if genistein regulates PR expression at the level of transcription, as does E_2, the effects of genistein and E_2 on PR gene promoter activity were compared by transient transfection assays. Parental hFOB cells, which lack endogenous ER expression, were co-transfected with a PR_A promoter-CAT reporter gene construct and an ER expression vector, encoding either ERα or ERβ. The ERs were FLAG-tagged at their N-termini, and Western blotting using an anti-FLAG antibody revealed similar protein levels of each ER isoform in cells 48 h after transfection (data not shown). Genistein and E_2 both stimulated the activity of PR_A through binding to either ER isoform (Fig. 2C). The ERα isoform, however, was a more efficient stimulator of PR_A transcription compared with ERβ in the presence of both ligands. The E_2 and genistein increased the PR_A promoter activity by a maximum of 8- and 4-fold, respectively, when acting with the ERα isoform, but only by a maximum of 3- and 2.5-fold, respectively, with the ERβ isoform. In summary, using conditions of equivalent ER concentrations, the ERα isoform displays a more

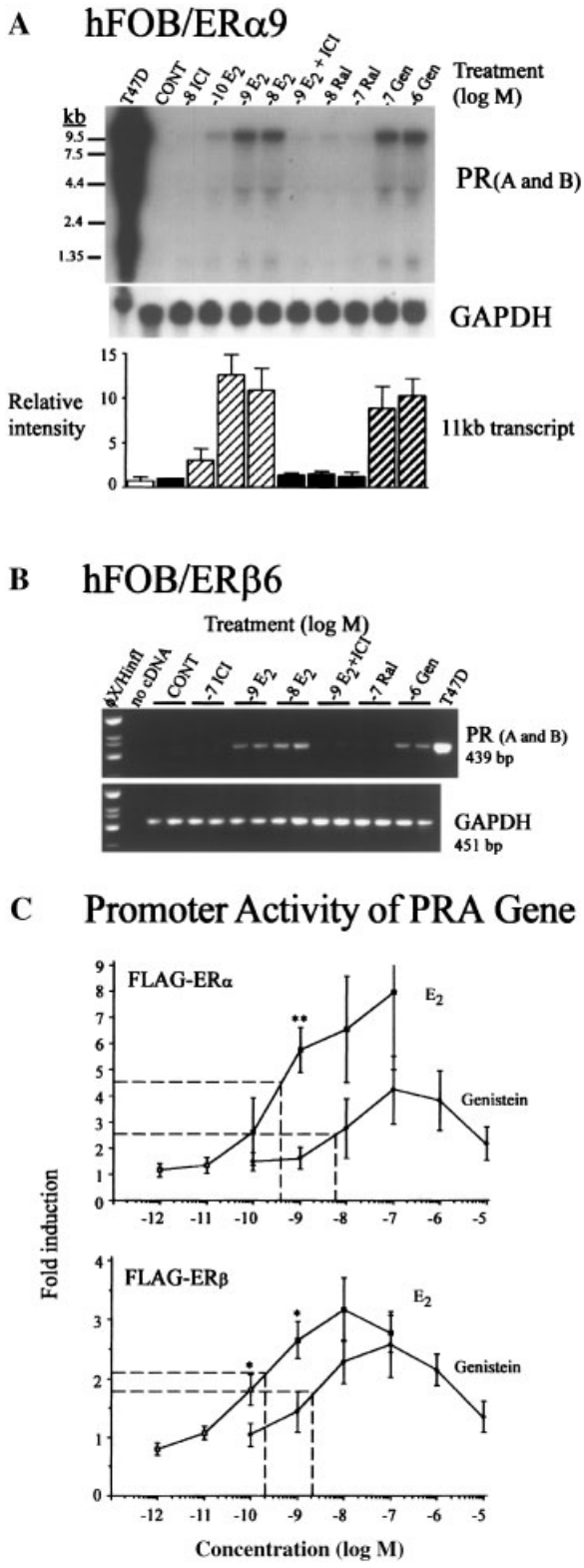

Fig. 2. Genistein and E_2, but not raloxifene, induce PR gene expression and promoter activity via ERα and ERβ. **Panel A**: Northern analysis of hFOB/ERα9 cells for both A and B isoforms of PR. Cells were pretreated with ICI 182,780 (ICI) prior to treatment for 24 h with genistein (Gen), E_2, raloxifene (Ral) or ICI at the indicated concentrations in DMEM-F12 + 0.1% (w/v) BSA medium. Densitometry of the major PR transcript of ~11 kb (normalized to GAPDH) is shown below, expressed relative to the level in cells treated with ICI alone. Values are the mean ± SEM of three separate experiments. **Panel B**: RT-PCR for PR (both A and B isoforms) and GAPDH in hFOB/ERβ6 cells continuously treated for 10 days in medium containing 10% (v/v) CS-FBS. T47D human breast cancer cells were used as a positive control for PR expression. **Panel C**: Stimulation of PR_A promoter activity by genistein and E_2 in the presence of ERα (**top**) and ERβ (**bottom**). The hFOB cells were transiently co-transfected with the appropriate ER expression vector encoding FLAG-tagged receptor together with the PR_A-CAT reporter gene construct PR-(464,1105)-CAT and CMV-luciferase. After pretreating with ICI, transfected cultures were stimulated with genistein or E_2 for 24 h in DMEM-F12 medium + 0.1% (w/v) BSA. Results are presented as fold induction of CAT activity compared to untreated control cultures, after normalizing with luciferase for transfection efficiency, and are the mean ± SEM of at least four separate experiments. A statistically significant difference between genistein and E_2 treatment at the same concentration is indicated by an asterisk (*).

potent action on the PR promoter activity than does the ERβ isoform. Further, E_2 was over 10-fold more potent than genistein at stimulating PR_A promoter activity irrespective of the ER isoform involved. The EC50 with ERα was, $E_2 = 4 \times 10^{-10}$ M, genistein $= 5.7 \times 10^{-9}$ M; while the EC50 with ERβ was $E_2 = 2 \times 10^{-10}$ M, genistein $= 2.2 \times 10^{-9}$ M. These data, using the transient transfection approach, are in general agreement with the regulation of endogenous PR gene expression observed in the above-described stably transfected hFOB/ER cell lines. These data demonstrate that genistein behaves as a weak E_2 agonist when signaling through either ERα or ERβ at the level of transcription (i.e., PR_A promoter activity).

Regulation of Versican Expression

Screening for possible estrogenic regulation of extracellular matrix constituents in hFOB/ERα9 cells revealed a marked stimulation of the mRNA levels for versican by E_2. Versican is a large chondroitin sulphate proteoglycan related to aggrecan and neurocan [Kimata et al., 1986; Zimmermann and Ruoslahti, 1989]. After a 24 h treatment period, both E_2 and genistein increased the versican mRNA expression in hFOB/ERα9 cells (Fig. 3A). Transcripts encoding two versican splice variants, designated V0 and V1 that differ in their glycosaminoglycan (GAG) attachment domains, were detectable by Northern blotting. A third splice variant, V2, was not induced by either E_2 or genistein in the hFOB/ERα9 or hFOB/ERβ6 cell lines (data not shown). Densitometry analysis showed that both genistein (10^{-7} and 10^{-6} M) and E_2 (10^{-9} to 10^{-8} M) produced a 2-fold increase in V0 and V1 mRNA levels relative to the ICI-treated control in the hFOB/ERα9 cells (Fig. 4B). Interestingly, treatment of hFOB/ERβ6 cells with genistein or E_2 from 24 h to 10 days, failed to modulate versican mRNA abundance (Fig. 3A,B). Raloxifene (10^{-8} and 10^{-7} M) had no effect on the expression of versican in either of the cell lines. Fig. 3C shows the results of RT-PCR using isoform-specific primers for V0 and V1. The data support the Northern blot analysis (Fig. 3A,B).

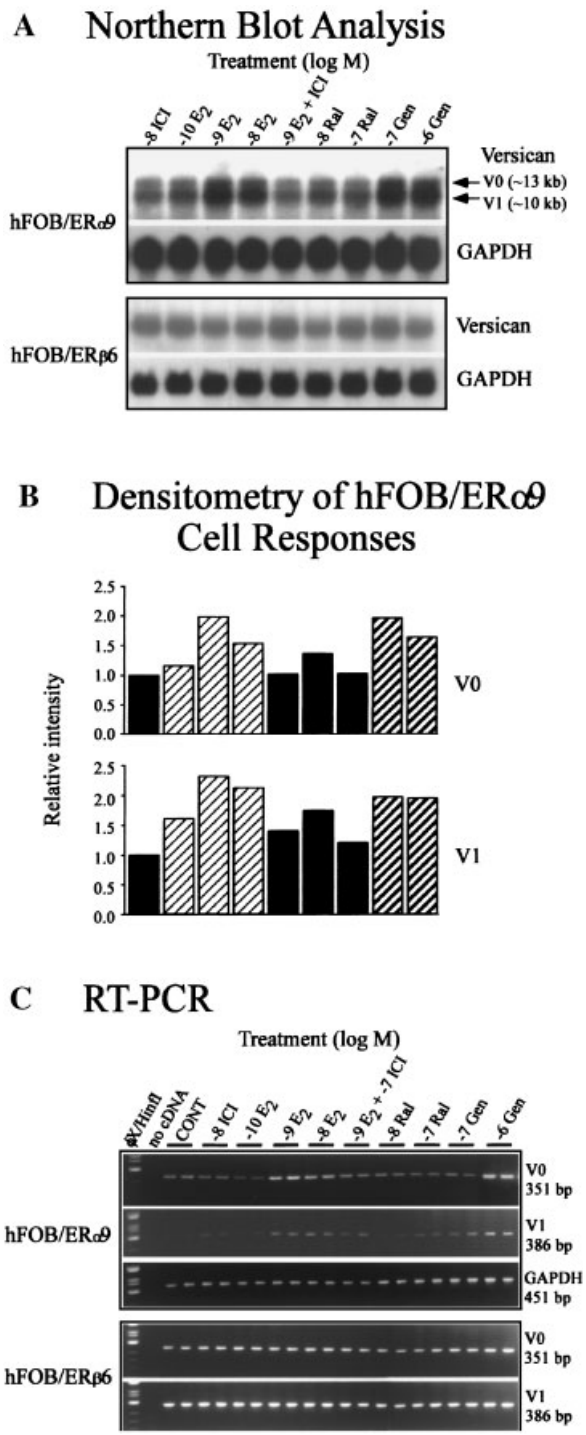

Fig. 3. Genistein and E_2 increase versican mRNA expression in hFOB/ERα9 cells but not in hFOB/ERβ6 cells. The hFOB/ERα9 cells were ICI pretreated before stimulation with genistein (Gen), E_2, raloxifene (Ral) or ICI for 24 h in DMEM-F12 medium + 0.1% (w/v) BSA. The hFOB/ERβ6 cells were treated with the same agents continuously for 10 days in medium + 10% (v/v) CS-FBS. **Panel A**: Total RNA was extracted and analyzed for versican expression by Northern blot hybridization using a full-length cDNA probe. **Panel B**: Densitometry of the V0 and V1 versican isoforms in hFOB/ERα9 cells from Panel A is shown relative to cells treated with ICI alone, after normalizing to the GAPDH level. A representative experiment, repeated two times with similar results, is presented. **Panel C**: RT-PCR analysis for the V0 and V1 splice variants of versican.

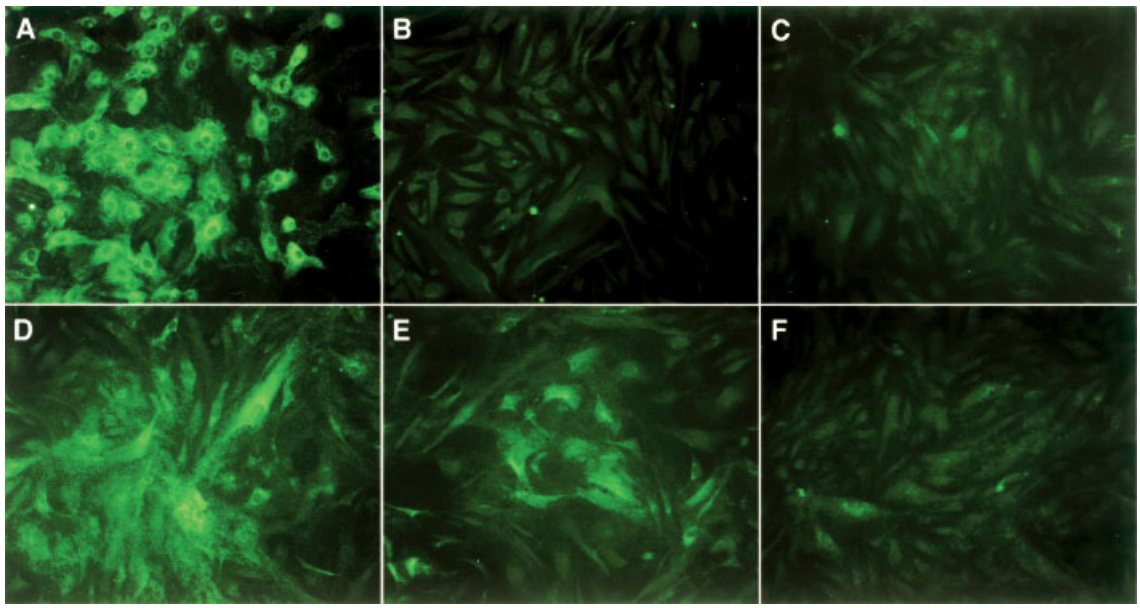

Fig. 4. Immunofluorescence detection of versican protein in MG63 and hFOB/ERα9 cells. Versican was detected by indirect immunofluorescence using an anti-human versican polyclonal antiserum and FITC-conjugated secondary antibody. MG63 human osteosarcoma cells were used as a positive control (**Panel A**). As a negative control, the cells were incubated with purified rabbit IgG instead of versican antiserum (**Panel B**). The hFOB/ERα9 cells were treated for 48 h in DMEM-F12 medium + 0.1% (w/v) BSA with either vehicle (**Panel C**), 10^{-9} M E_2 (**Panel D**), 10^{-6} M genistein (**Panel E**) or co-treated with 10^{-9} M E_2 and 10^{-7} M ICI (**Panel F**). Magnification ×50.

The E_2 and genistein regulation of versican protein concentration in hFOB/ERα9 cells was then assessed by indirect immunofluorescence. Staining of versican protein human osteosarcoma cells (Fig. 4A) served as a positive control with widespread and intense staining. With no steroid treatment, the hFOB/ERα9 cells showed a minority of the cells with perinuclear and punctate cytoplasmic staining (Fig. 4C). Treatment of hFOB/ERα9 cells with E_2 (Fig. 4D) or with genistein (Fig. 4E) for 48 h increased both the staining intensity and the fraction of positively stained cells. Co-treatment of these cells with E_2 plus a 100-fold molar excess of ICI 182,780 prevented the E_2-mediated increase in versican protein staining, results which support the ICI antagonist effect at the mRNA level.

Responses of Other Bone Matrix Protein Genes

The effects of genistein, E_2, and raloxifene on the expression of the osteoblast marker genes, *AP*, *OP*, and *ON*, were also investigated in the two hFOB/ER cell lines. As previously reported in hFOB/ERα9 cells [Robinson et al., 1997; Waters et al., 2001], E_2 at the highest concentration tested (10^{-8} M) in hFOB/ERα9 (Fig. 5A) stimulated the level of the 2.5 kb mRNA of AP. Genistein at 10^{-6} M, but not raloxifene, also increased the AP mRNA expression to a similar degree as E_2. Conversely, both genistein and E_2 inhibited the OP steady state mRNA levels (1.6 kb) in hFOB/ERα9 cells. The steady state mRNA levels for the two ON transcripts at 3.0 kb and 2.2 kb were unaffected by any of the ER ligands tested. As shown in Fig. 5B, the expression of none of these genes in hFOB/ERβ6 cells was modulated by genistein, E_2, or raloxifene, following prolonged treatment for 4–10 days.

Regulation of Interleukin-6 Synthesis

Interleukin-6 synthesis was also examined for possible regulation by E_2 and genistein. The constitutive synthesis of IL-6 by both hFOB/ERα9 and hFOB/ERβ6 cells has previously been demonstrated to be suppressed by E_2 [Waters et al., 2001], as has both IL-6 production by endometrial cells expressing only ERβ [Deb et al., 1999] and cytokine-stimulated IL-6 production by osteoblast lineage cells [Passeri et al., 1993]. In agreement with these reports, E_2 treatment of hFOB/ER cells, expressing either ERα or ERβ, caused an inhibition of IL-6 synthesis as measured by ELISA of conditioned

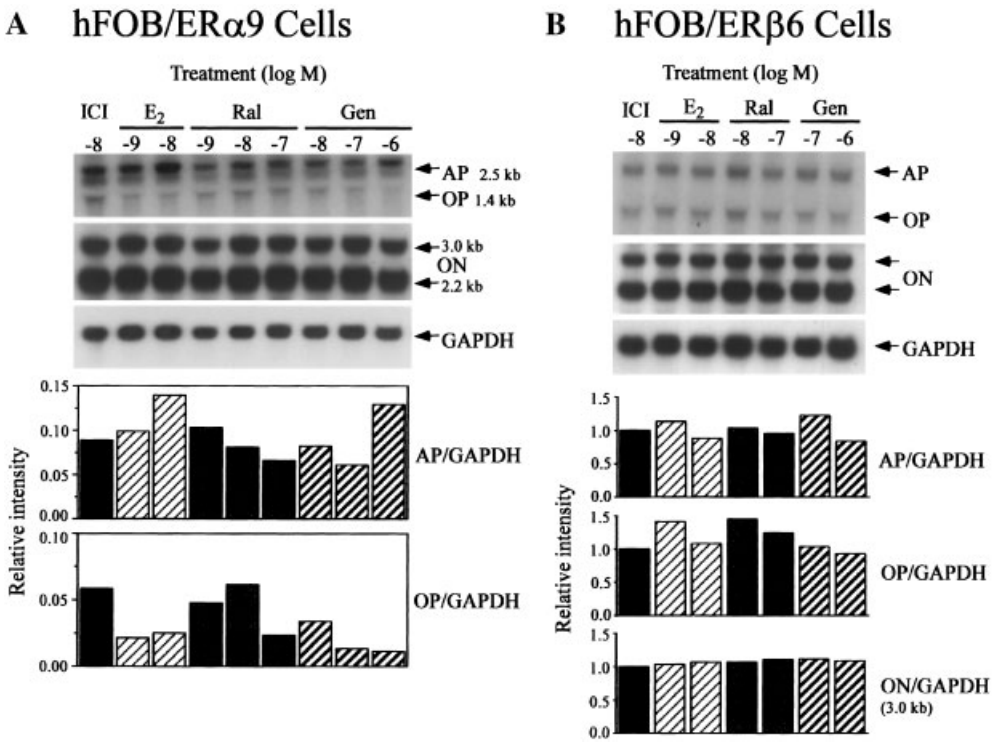

Fig. 5. Analysis of the alkaline phosphatase (AP), osteopontin (OP), and osteonectin (ON) mRNA expression by genistein, E_2 and raloxifene in hFOB/ER cell lines. The hFOB/ER cells were treated as described in Figure 4 and steady state mRNA levels in total RNA analyzed by Northern blotting. **Panel A**: hFOB/ERα9 cells. **Panel B**: hFOB/ERβ6 cells. Membranes were sequentially hybridized to cDNA probes for AP, OP, ON, and then for GAPDH to correct for RNA loading. The band intensities were determined by densitometry, normalized for GAPDH, and are presented as mean of two separate experiments expressed relative to levels in cells treated with ICI alone.

medium (Fig. 6). The synthesis of IL-6 was suppressed by a similar magnitude in both cell lines with E_2 at 10^{-8} M decreasing IL-6 levels to 50–60% of the ICI-treated control. Again, genistein acted as an E_2 agonist and significantly decreased IL-6 synthesis via both ERα and ERβ. Interestingly, the suppression of IL-6 by genistein was both of greater magnitude and occurred at lower concentrations in the hFOB/ERβ6 cell line than in hFOB/ERα9 cells, correlating with the higher affinity of genistein for ERβ over ERα [Kuiper et al., 1998]. The genistein-mediated inhibition of IL-6 synthesis in both hFOB/ER lines was reversed by co-administration of ICI 182,780, verifying that the inhibitory effects were indeed ER dependent. Although raloxifene significantly reduced IL-6 production in the hFOB/ERα9 cells, the response was minor and was not dose-dependent. Raloxifene did not affect IL-6 levels in hFOB/ERβ6 cells. These data support the notion that the regulatory actions of E_2 and genistein are virtually identical once the higher level of genistein is achieved, that genistein is a less potent estrogenic compound, and that the particular ER isoform dictates the overall response to any steroid analogue.

Cell Proliferation

Since the bFGF stimulates osteoblast proliferation through an intracellular tyrosine kinase domain on the FGF receptor and since genistein is an inhibitor of tyrosine kinase activity (IC50 ~2.6 μM) [Akiyama et al., 1987], the effect of genistein on both the basal and bFGF-stimulated proliferation of the ER-negative hFOB cells was examined and compared to the effects of E_2 and raloxifene. As expected, for 5 days of treatment, raloxifene (10^{-8} to 10^{-7} M) or E_2 (10^{-10} and 10^{-8} M) was shown to have no effect on proliferation in the absence or presence of bFGF (Fig. 7B) since the hFOB cells do not express endogenous ER. Genistein at 10^{-7} M was also without effect, but higher concentrations did show an inhibitory effect (Fig. 7A). Genistein at 10^{-6} M reduced the basal proliferation of hFOB cells by ~20% and 10^{-5} M produced an 80% reduction in both the basal

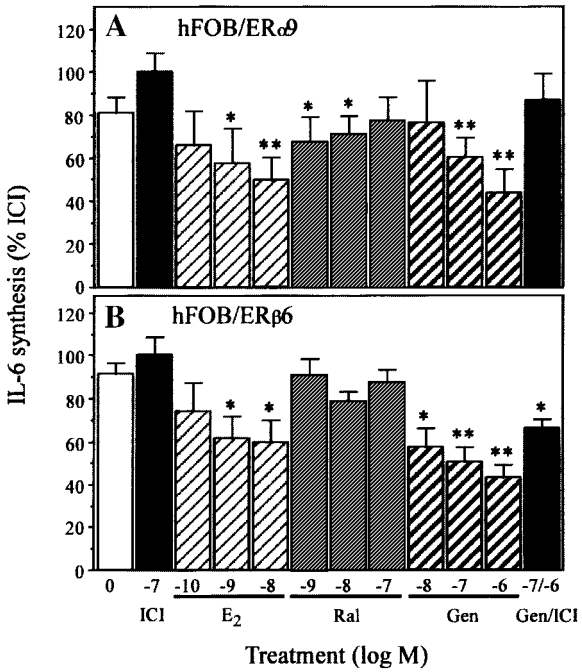

Fig. 6. Dose-dependent inhibition of IL-6 protein levels by genistein and E_2, but not by raloxifene, in hFOB/ER cell lines. Cells were treated for 4 days with genistein (Gen), E_2, raloxifene (Ral), and/or ICI 182,780 (ICI) at the concentrations indicated in medium containing 10% (v/v) CS-FBS. Conditioned medium harvested on day 4 was assayed for IL-6 using an ELISA. **Panel A**: hFOB/ERα9. **Panel B**: hFOB/ERβ6 cells. The data are the mean ± SEM of three separate experiments. Asterisks denote a statistically significant difference compared to treatment with ICI alone (100%).

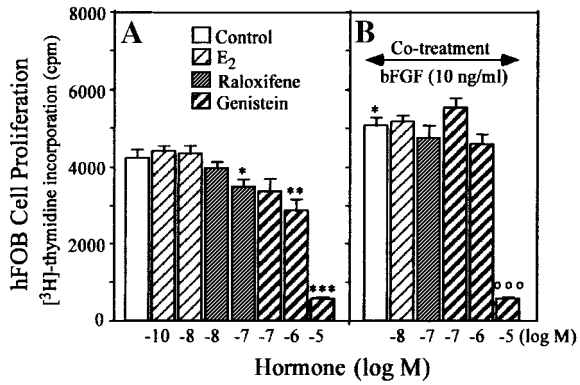

Fig. 7. Receptor independent effects of genistein, E_2, and raloxifene on the hFOB cell proliferation. Cells pretreated with ICI were then treated for 5 days with genistein, E_2 and raloxifene in the absence **(Panel A)** or presence **(Panel B)** of bFGF (10 ng/ml) in medium supplemented with 1% (v/v) CS-FBS. Cells were labeled with [^{3}H]-thymidine for the final 20 h of treatment and the incorporation of radioactivity into trichloroacetic acid-precipitable material was determined as a measure of DNA synthesis. Each treatment was added to six wells. Results are given as the mean ± SEM of a representative experiment repeated two times. Significant differences between treatments and the control-treatment are indicated by asterisks and significant differences between bFGF alone and bFGF co-treatments are indicated by $^{000}P < 0.001$.

(Fig. 7A) and bFGF-stimulated (Fig. 7B) proliferation.

The inhibitory effect of genistein on proliferation of these ER deficient hFOB cells was shown to be largely due to reduced cell proliferation rather than increased cytotoxicity as determined by the Trypan blue exclusion method. At the end of the 5-day treatment period, cultures administered with 10^{-5} M genistein had approximately twice the number of non-viable cells as untreated cultures or cultures treated with 10^{-8} M E_2 or 10^{-7} M raloxifene. In contrast, over the same time period, the number of viable cells increased only 1.5-fold in the presence of 10^{-5} M genistein, whereas viable cell number had increased by ~15-fold for the other conditions (data not shown). Thus, the dramatic inhibition of DNA synthesis by genistein, as quantitated in the [^{3}H]-thymidine incorporation assay, was almost completely due to reduced cell division rather than to increased cell death.

DISCUSSION

The effect of the natural isoflavone, genistein, on the activity of osteoblast cell lines that express either ERα or ERβ, was compared to that of E_2 and a SERM (raloxifene). In almost every instance, genistein behaved as a weak ER agonist. In addition, genistein displayed a receptor independent function by its effects on cell proliferation when added at high concentrations. Both E_2 and genistein induced PR gene expression and promoter activity, stimulated the steady state mRNA levels for AP and versican, and inhibited *OP* gene expression and IL-6 protein production. The levels of IL-6 and PR mRNA were regulated in both hFOB/ERα9 and hFOB/ERβ6 cells, whereas these levels for AP, OP, and versican were only regulated in the hFOB/ERα9 cells. Interestingly, raloxifene failed to regulate any of the parameters in the OB cells expressing either ER isoform. Differences in gene regulation between the two cell lines to E_2 and genistein may reflect unequal ER expression, which we suspect is somewhat lower in the ERβ cell line. Nonetheless, this does not prevent comparison of the effects of E_2 with genistein and raloxifene in each of the cell lines separately, as presented here.

In general, genistein concentrations of 10- to 100-fold higher than for E_2 were required to observe responses of comparable magnitude. Similarly, using transient transfection assays, maximal activity of the PR_A promoter was achieved with an approximately 10-fold higher concentration of genistein compared to E_2, however, the maximal promoter activity was still lower with genistein. These findings therefore correspond, in part, to the reported ~20-fold and 3-fold lower binding affinity of genistein for ERα and β, respectively, compared to E_2 [Kuiper et al., 1998]. An additional explanation for the lower potency of genistein may include differences in activity of the two genistein-complexed receptor isoforms towards the endogenous PR promoters compared to the transfected PR_A promoter fragment.

The suppression of OP mRNA by genistein and E_2 in hFOB/ERα9 cells coincides with a previous report demonstrating down-regulation of bone matrix protein expression, including OP, in bones of E_2-treated rats [Turner et al., 1990]. In addition, E_2 has been shown to inhibit both osteocalcin synthesis [Robinson et al., 1997] and mineralized extracellular matrix deposition [Waters et al., 2001] in hFOB/ERα9 cells. OP is an acidic glycoprotein important for cell attachment and considered a late marker of osteoblastic differentiation and an early marker of mineralization [Robey and Boskey, 1996]. Overall, these findings suggest that in this osteoblast cell line, ER agonists including genistein, arrest osteoblast differentiation prior to the onset of both mineralization and the expression of late differentiation markers. Although the precise role of OP in bone is unclear, OP has been shown to be required for osteoclast-mediated bone resorption: binding of extracellular OP to the integrin $\alpha_v\beta_3$ on the surface of osteoclasts activates cytoskeletal reorganization in podosomes, stimulating cell motility and resorption [Ross et al., 1993]. Since OP can induce osteoclastic bone resorption, at least in vitro, the ER-mediated inhibition of OP expression observed here may therefore contribute to the overall anti-resorptive effects of estrogen. It remains unknown whether the inhibition of OP expression in osteoblasts occurs by a direct suppression of gene transcription or indirectly as the result of arrested osteoblast differentiation.

Both genistein and E_2 stimulated the mRNA levels for AP in hFOB/ERα9 cells, in accordance with previous reports of the effects of estrogen on osteoblastic cells by our laboratory and others [Robinson et al., 1997]. AP is generally considered to be an earlier marker of osteoblast differentiation than OP, the levels of which increase in primary osteoblast cultures following cessation of cell division and continue to rise thereafter [Owen et al., 1990]. Consequently, if ER agonists do arrest differentiation in hFOB/ERα9 cells, they do so at a stage subsequent to AP expression and before the induction of OP.

The synthesis of IL-6 protein by hFOB/ER cell lines was inhibited by genistein and estrogen acting through both ER isoforms. The effective genistein concentration required to inhibit IL-6 was lower in the ERβ expressing osteoblasts compared to the ERα containing cells which probably reflects the greater affinity of genistein for ERβ [Kuiper et al., 1998]. The anti-resorptive effects of high-dose isoflavones such as genistein in vivo, may be due to the inhibition of multinucleated osteoclast formation and activity, as reported for high concentrations (>1 μM) of the synthetic isoflavone, ipriflavone, and select metabolites when added to bone organ and unfractionated bone cell cultures in vitro [Notoya et al., 1993; Giossi et al., 1996]. Since enhanced IL-6 production by osteoblast lineage cells has been strongly implicated in causing osteoclast differentiation and thus increased bone resorption and rate of bone loss in estrogen deficiency [Jilka et al., 1992; Passeri et al., 1993], the genistein-mediated suppression of IL-6 may be an important additional mechanism explaining the anti-resorptive effects of isoflavones. More recently, genistein has been reported to increase the ratio of OPG:OPGL (RANKL) expression by osteogenic stromal cells, which would also be expected to suppress osteoclastogenesis [Yamagishi et al., 2001]. The IL-6 promoter is suppressed by E_2-ER complexes through the binding of the transcription factors, NF-κB and C/EBPβ [Stein and Yang, 1995]. It would therefore be of interest to determine whether genistein-activated ERα and ERβ suppress transcription of the *IL-6* gene via interactions with the same set of factors and response elements.

The identification of versican (PG-M in chickens) as a target for estrogen regulation represents another novel finding of this investigation. Versican is a large, multidomain, chondroitin sulphate-linked proteoglycan, related to aggrecan, brevican, and neurocan [Kimata

et al., 1986; Zimmermann and Ruoslahti, 1989]. We have previously demonstrated that estrogen significantly displays mineralized matrix deposition in the hFOB/ERα9 cell line, but not in hFOB/ERβ6 cells [Waters et al., 2001]. We speculate that one mechanism for this delay is the ER regulation of different bone matrix protein genes. Versican has been shown to be predominantly expressed during embryonic skeletal development, and later in cartilage and unmineralized embryonic bone, as well as in numerous adult connective tissue cell types [Shinomura et al., 1990; Bode-Lesniewka et al., 1996]. It is possible that the estrogen inhibition of mineralization of bone matrix is due to the altered matrix proteins in the matrix due to the action of estrogen, fibroblasts, keratinocytes, and arterial smooth muscle cells [Shinomura et al., 1990; Bode-Lesniewka et al., 1996]. During embryonic skeletal development, versican is localized to prechondrogenic condensations and subsequently to the cartilage core and immature bone, although versican is absent from mature cartilage [Kimata et al., 1986; Shinomura et al., 1990]. It is unknown whether versican is present in adult human bone although the protein is synthesized and secreted by MG63 human osteosarcoma cells [LeBaron et al., 1992].

Versican has been shown to inhibit cell attachment to matrix as well as modulate cell motility and differentiation through binding to hyaluronan, type I collagen and fibronectin, [Perissinotto et al., 2000]. In addition, the GAG side chains of versican can bind chemokines, modifying their activity. Since the function of versican in cartilage and other connective tissues remains obscure, and its presence in adult mineralized bone is unknown, it is difficult to speculate on the possible physiological significance of the estrogenic regulation of versican synthesis in bone. Alternatively, versican expression by the hFOB/ERα9 and hFOB/ERβ6 cells may be a consequence of their fetal origin, implying a role for estrogen in early stages of bone formation.

Both E_2 and genistein stimulated the expression of the same splice variants of versican, designated V0 and V1, which are the isoforms also expressed by fibroblasts [Dours-Zimmermann and Zimmerman, 1994]. A third splice variant that has been detected in brain, V2, was neither constitutively expressed in hFOB/ER cells nor induced by the ER ligands. Versican expression in arterial smooth muscle cells has been shown to be stimulated by platelet-derived growth factor (PDGF) and TGFβ, and genistein has been shown to antagonize the effect of PDGF by inhibiting the PDGF receptor tyrosine kinase activity [Schonherr et al., 1997]. This highlights the dual mechanism of genistein action on cellular responses, wherein the effects of low genistein concentrations are likely to be ER-mediated whereas effects at high concentrations (>1 μM) may be due to inhibition of tyrosine kinases (the IC50 for the EGFR tyrosine kinase is 2.6 μM). In this regard, in our study, the proliferation of the ER-negative hFOB cell line was potently inhibited by 10^{-5} M genistein whereas lower concentrations of genistein as well as ER-saturating concentrations of both E_2 and raloxifene were without effect.

Perhaps surprising was the lack of response of the hFOB/ER cell lines to raloxifene for any of the parameters examined here. The bioactivity of the HPLC-purified raloxifene preparation was verified by antagonism of E_2-stimulated MCF-7 cell growth. Thus, at concentrations which should saturate the ER, raloxifene failed to affect either hFOB/ER cell line, despite the fact that the actions of E_2 and raloxifene on bone metabolism in vivo and on osteoclast lineage cells in vitro are very similar [Bryant et al., 1999]. Whereas some effects of raloxifene on osteoblastic cells are similar to those produced by E_2, differences have also been reported. For example, the induction of TGFβ_3 expression by raloxifene in intact bone and cultured bone cells is also produced by metabolites of E_2 but only weakly by E_2 itself [Yang et al., 1996]. Moreover, raloxifene, but not E_2 or idoxifene, antagonized ERE-dependent transcriptional activity in osteoblastic cells [Nuttall et al., 2000]. Thus, it is possible that raloxifene and E_2 may differentially regulate ER target genes in osteoblasts and raloxifene alone may regulate a unique subset of genes not affected by E_2.

In vivo treatment with isoflavones, in contrast to estrogen and SERMs, has little effect on the high bone turnover rate that is characteristic of sex steroid deficiency and reportedly enhances the rate of bone formation [Gennari et al., 1997; Arjmandi et al., 2000]. Differences in behavior between genistein and E_2 could therefore be due to a differential regulation of ER-dependent mechanisms in bone cells. Using human osteoblast cell lines that over-express

either ERα or ERβ, we have shown that the natural isoflavone, genistein, functions as an ER agonist. However, at high concentration (10 μM) the effect of genistein diverges from those of E_2 with regard to ER dependency and are speculated here to be due to the inhibition of tyrosine kinases. In general, our data demonstrates that genistein mimics the ER-mediated effects of E_2 in osteoblasts. Although we cannot exclude the possibility that responses other than those examined may be differentially regulated by genistein and E_2 acting via ER, divergent signaling of non-genomic and nuclear ER-independent mechanisms may instead account for the effects in vivo. Further investigation is necessary to fully define the actions of genistein on osteogenic cells.

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