Age-related changes in effects of insulin-like growth factor I on human osteoblast-like cells

Patricia Y. D'AVIS, Chester R. FRAZIER, Jay R. SHAPIRO and Neal S. FEDARKO*

Division of Geriatrics, Department of Medicine, Room 5A-50 JHAAC, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, U.S.A.

The role of insulin-like growth factor I (IGF-I) in extracellular matrix metabolism was studied in both proliferating and confluent human osteoblast-like cultures derived from donors of different ages. In proliferating cultures, recombinant human (rh)IGF-I was found to increase the incorporation of [^{3}H]thymidine in a dose- and age-dependent manner. To study cell proliferation dynamically, continuous growth curves with and without rhIGF-I were modelled by a modified logistic function. Increasing doses of rhIGF-I decreased the lag time and maximal growth rates, whereas plateau values decreased only at the highest dose (100 ng/ml). In post-proliferative cell strains, rhIGF-I (0.1–100 ng/ml) increased levels of type I collagen, biglycan and decorin, and to a smaller extent fibronectin and thrombospondin, whereas it decreased the levels of hyaluronan and a versican-like proteoglycan when protein and proteoglycan metabolism were followed by steady-state radiolabelling with [^{3}H]proline, [^{3}H]glucosamine or [^{35}S]sulphate. These responses to rhIGF-I were found to be age-dependent, with osteoblast-like cells derived from younger patients being more responsive to rhIGF-I. When extracellular matrix turnover was analysed by pulse–chase experiments, rhIGF-I had no effect. The steady-state levels of collagen, decorin, hyaluronan and a versican-like proteoglycan for bone cells treated with rhIGF-I on day 7 in culture were equivalent to levels of these matrix components in untreated osteoblasts grown for 14 days. These results are consistent with rhIGF-I's altering cellular proliferative capacity and matrix synthesis, causing a change in the osteoblast differentiated state.

INTRODUCTION

Insulin-like growth factor I (IGF-I) is a polypeptide that possesses a pro-insulin-like chemical structure with insulin-like and cell growth-promoting properties. Studies both *in vivo* and *in vitro* suggest that IGF-I might play a role in bone metabolism. IGF-I administered *in vivo* to rats increased bone formation [1,2]. A possible role for IGF-I in the pathogenesis of the increased bone turnover that occurs early in ovarian hormone deficiency was proposed on the basis of work in an ovariectomized rat model system [3–7]. In humans, increasing age has been associated with a linear decline in human skeletal content of IGF-I [8] and a decreasing molar ratio of IGF-I to IGF-binding protein 3 in serum [9]. The most frequently reported effect of IGF-I on osteoblast-like cells *in vitro* has been the stimulation of cellular proliferation. Proliferation was increased by IGF-I in MC3T3-E1 cells [10], the human OHS-4 cell line [11], the human TE89 cell line [12], MG63 osteosarcoma cells [13], SAOS-2 cells [14], UMR 106-06 cells [15] and primary fetal rat calvaria cells [16]. IGF-I stimulated, in a dose- and time-dependent manner, the specific activity of creatine kinase (a marker of skeletal cell division) in both female and male rat calvarial bone cells, in ROS 17/2.8 cells and in epiphyseal cartilage cell cultures [17].

Most osteoblasts in bone are not actively proliferating cells, so the biological relevance of stimulated proliferation *in vitro* is unclear. The present study was undertaken to follow the effects of IGF-I on proliferating human osteoblast-like cells and on bone matrix component synthesis and turnover in non-proliferating cells. Previous work with normal human osteoblast-like cell systems has shown an age-related decrease in maximal growth rates [18], extracellular matrix component synthesis [19,20] and responsiveness to growth factors and hormones [21]. To investigate the capacity of IGF-I to affect the pathophysiology of age-related decreases in bone, the effects of recombinant human (rh)IGF-I on human osteoblasts derived from donors of different ages were studied.

MATERIALS AND METHODS

Cell culture

Biopsies for the isolation of osteoblastic cells were obtained in accordance with approved procedures of the Institutional Review Board, Johns Hopkins Bayview Medical Center. Bone specimens were obtained from iliac crest bone intended for bone graft or from uninvolved distal portions of the femoral neck removed during hip arthroplasty. Human trabecular osteoblast-like cells were cultured by the method of Gehron Robey and Termine [19,22]. Briefly, 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 (Advanced Biofactures) for 2 h at 37 °C. Minced bone fragments were plated in 100 cm^2 sterile culture dishes. Cell strains obtained in this fashion were characterized as osteoblast-like by demonstrating a high alkaline phosphatase activity (between 0.2 and 0.6 unit/10^6 cells) [23] as well as the induction of intracellular cAMP levels in response to parathyroid hormone [24] and the formation of an extracellular matrix that contained components found *in vivo* and that mineralized in a physiological fashion [19,25]. Osteoblast-like cells were grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, 2 mM glutamine and 4.5 g/l glucose. Cells from explant culture were harvested with trypsin/EDTA and studied in passage 1 or 2. Tissue culture grade

Abbreviations used: IGF-I, insulin-like growth factor I; rh, recombinant human.

* To whom correspondence should be addressed.

rhIGF-I (Collaborative Biomedical Products) was reconstituted in 10 mM sodium acetate buffer, pH 6.0.

[^{3}H]Thymidine incorporation

The influence of rhIGF-I on the incorporation of [^{3}H]thymidine into DNA was assayed by labelling osteoblast-like cells for 12 h with 0.5 μCi/ml [^{3}H]thymidine in the presence of various concentrations of rhIGF-I [23]. After incubation, plates were placed on ice and cell monolayers were rinsed five times with chilled Tris-buffered saline [150 mM NaCl/20 mM Tris (pH 7.6)]. All subsequent steps were performed at 4 °C. The plates were then treated with 10 % (w/v) trichloroacetic acid containing 10 mM thymidine for 15 min at 4 °C. After a second 15 min wash with cold trichloroacetic acid, the cell monolayer was rinsed twice with ethanol. The trichloroacetic acid-precipitable material was solubilized by adding 0.5 M NaOH to the dried wells. Levels of incorporation were quantified by liquid-scintillation counting (Ecoscint; National Diagnostics, Atlanta, GA, U.S.A.) [23].

Growth curve analysis

Cell proliferation kinetics were determined in triplicate with a Crystal Violet assay [18,26]. Briefly, cells were fixed with 1 % glutaraldehyde in PBS [10 mM NaH_2PO_4/150 mM NaCl (pH 7.2)] for 15 min, stained for 30 min with a solution of 0.02 % Crystal Violet and rinsed twice with distilled deionized water; the stain bound to cells was extracted overnight at 4 °C in 70 % (v/v) ethanol. Absorbance was determined at 578 nm. The growth curves were modelled by fitting a Zweitering modified logistic to each set of observed absorbance–time values and solving for the biologically relevant parameters upper asymptote, which reflects the maximum cell density on confluence, maximal growth rate (μ_m) and lag time [18]. The logistic fit is calculated by nonlinear regression with a Marquardt algorithm and DeltaGraph (DeltaPoint, Inc., Monterey, CA, U.S.A.).

Extracellular matrix component analysis

Cultures were incubated for 24 h in antibiotic-free, serum-free medium and then radiolabelled at steady state for 24 h with Dulbecco's modified Eagle's medium (low-sulphate) supplemented with dialysed 2 % (v/v) fetal bovine serum/50 μg/ml ascorbic acid/2 mM glutamine. Cells were radiolabelled with 10 μCi/ml [2,3,4,5-^{3}H]proline (100 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.) for [^{3}H]collagen, [^{3}H]-thrombospondin and [^{3}H]fibronectin analysis; with 100 μCi/ml carrier-free $H_2{}^{35}SO_4$ (1100 Ci/mmol, New England Nuclear) for [^{35}S]-labelled proteoglycan analysis; or with 10 μCi/ml [6-^{3}H]glucosamine (30 Ci/mmol, New England Nuclear) for [^{3}H]hyaluronan analysis. All labelling was done in triplicate. Cell numbers were determined on parallel cultures in triplicate at the end of the labelling period by use of a Coulter counter Model ZM (Coulter Electronics Inc., Hileah, FL, U.S.A.), after trypsin treatment. At the end of 24 h labelling time the medium was aspirated, the monolayer was rinsed twice with Tris-buffered saline, pH 7.6, and the washes were added to the medium and taken as the 'medium pool'. The 'cell layer' pool was generated by overnight extraction at 4 °C with a Tris buffer, pH 7.4, containing 4 M guanidinium chloride, 5 mM benzamidine, 10 mM *N*-ethylmaleimide, 0.1 M 6-aminohexanoic acid, 1 mM PMSF and 2 % (v/v) Triton X-100. Aliquots of the medium and cell layer pools were then taken for specific matrix component analysis (see below). Values determined for radiolabel incorporation were corrected for cell number.

Total radiolabel incorporation into protein was determined by descending chromatography on paper of aliquots from the pools developed overnight in n-butanol/acetic acid/1 M ammonia (2:3:2) [27]. Unincorporated radiolabel rapidly migrated from the origin, whereas polymer-bound radioactivity remained at the origin segment. After 18 h of development, the origin segment was excised and radioactivity counted in Betafluor liquid-scintillation fluid (National Diagnostics, Atlanta, GA, U.S.A.).

Levels of [^{3}H]collagen were determined as [^{3}H]hydroxyproline after sample precipitation, acid hydrolysis and descending chromatography on paper in 95 % ethanol/15 M NH_4OH (19:1) essentially as described [28]. [^{35}S]Sulphate incorporation into a versican-like proteoglycan [29], biglycan and decorin were determined by HPLC desalting and resolution on SDS/PAGE [3–15 % (w/v) gel gradient] exactly as described [30]. The incorporation of label into hyaluronan was determined by chromatography of an aliquot of the [^{3}H]glucosamine-labelled pool before and after digestion with *Streptomyces* hyaluronidase (ICN Biochemical) (3.3 turbidity-reducing units/ml in 0.02 M acetate buffer, pH 5.0) on a 1.0 cm × 25 cm ToyoPearl TSK-GEL HW 40 (S) column equilibrated in the formamide buffer system [31].

Steady-state levels of [^{3}H]thrombospondin and [^{3}H]fibronectin were determined by an immunoprecipitation assay, exactly as described [20]. Briefly, aliquots of medium and cell layer pools from cultures labelled with [^{3}H]proline were incubated sequentially with GammaBind C agarose (Genex Corporation), normal rabbit serum IgGs bound to GammaBind C agarose (to remove proteins binding non-specifically), and a third sweep with specific antibodies against thrombospondin and fibronectin (human) from Chemicon (Temecula, CA, U.S.A.). Each incubation was for 1 h at 4 °C and was terminated by centrifugation at 7000 ***g*** for 5 min. Immunoprecipitated [^{3}H]thrombospondin and [^{3}H]fibronectin were subjected to PAGE [4–20 % (w/v) gel gradient], fluorography, excision of the region of the gel corresponding to the labelled band, solution of the gel slice in 30 % (w/v) H_2O_2 for 12 h at 37 °C and resuspension in Ecoscint scintillation cocktail for liquid-scintillation counting.

RESULTS

IGF-I and osteoblast proliferation

The effect of rhIGF-I on [^{3}H]thymidine incorporation was determined on human osteoblastic strains derived from healthy female donors of different ages (Figure 1). In the absence of rhIGF-I, the bone cells derived from the 14-year-old were consistently found to incorporate radiolabel at levels 2–2.5-fold that of the bone cell strain derived from the 90-year-old. When different concentrations of rhIGF-I were added to the cells, an age-dependent dose response was observed for all three strains. A dose of 50 ng/ml rhIGF-I in the bone cell strain derived from the 90-year-old produced an increase in [^{3}H]thymidine incorporation equivalent to a dose of 0.1 ng/ml in the cell strains derived from younger bone.

[^{3}H]Thymidine incorporation provides a static measure of DNA synthesis. To study osteoblast proliferation dynamically, growth curves (Figure 2) were modelled by a modified logistic function. The Crystal Violet method of profiling cell number was found to be highly correlated with cell number as determined by trypsin/EDTA dissociation of the monolayer and quantification of the single cell suspension with a Coulter counter (Figure 2a, inset). Age of donor did not alter the high correlation of Crystal Violet absorbance values with cell number. The addition of rhIGF-I caused changes in growth curve profiles in osteoblast-like cells derived from both young (Figure 2a) and old (Figure

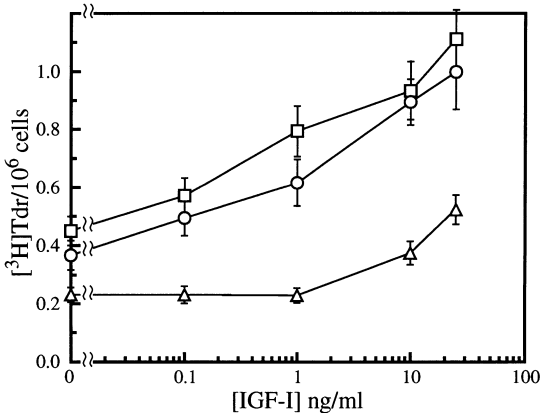

Figure 1 Stimulation by IGF-I of [^{3}H]thymidine incorporation by normal human osteoblasts

Osteoblasts derived from normal bone specimens of three female donors of different ages [14 (□), 40 (○), and 90 (△) years old] were seeded at 20000 cells/cm^2 and grown for 5 days. On the sixth day, cultures were radiolabelled with 2 μCi/ml [^{3}H]thymidine with or without various concentrations of IGF-I. [^{3}H]Thymidine incorporation into DNA was determined after 24 h as trichloroacetic acid-precipitable material. The [^{3}H]thymidine radioactivity was normalized on a 10^6 cell basis.

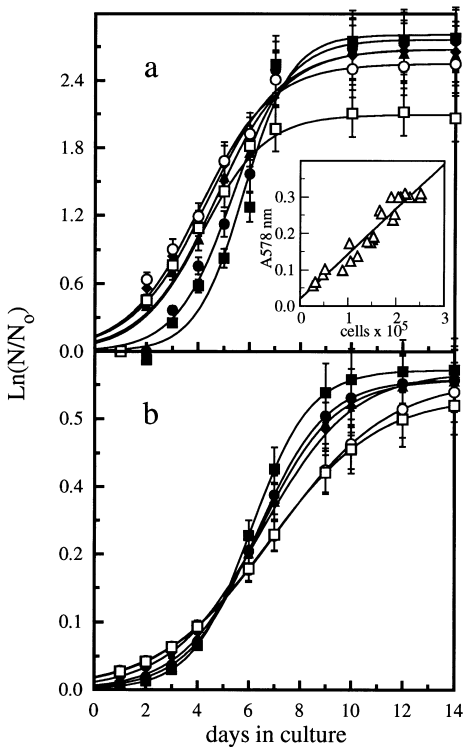

Figure 2 Effects of IGF-I on osteoblast growth profiles

Osteoblasts derived from normal bone specimens from donors age 4 (**a**) or 90 years (**b**) were seeded at 20000 cells/cm^2 and grown in different concentrations of rhIGF-I for 2 weeks. At each time point wells were fixed in triplicate for staining with Crystal Violet. The absorbance values for ethanol-solubilized Crystal Violet were fitted to a modified logistic curve as described in the Materials and methods section. Concentrations of IGF-I (ng/ml) were as follows: ■, 0; ●, 0.1; ▲, 1; ◆, 10; ○, 50; □, 100. Inset: Crystal Violet absorbance values plotted against the corresponding cell numbers determined by trypsin/EDTA dissolution of the monolayer on parallel wells.

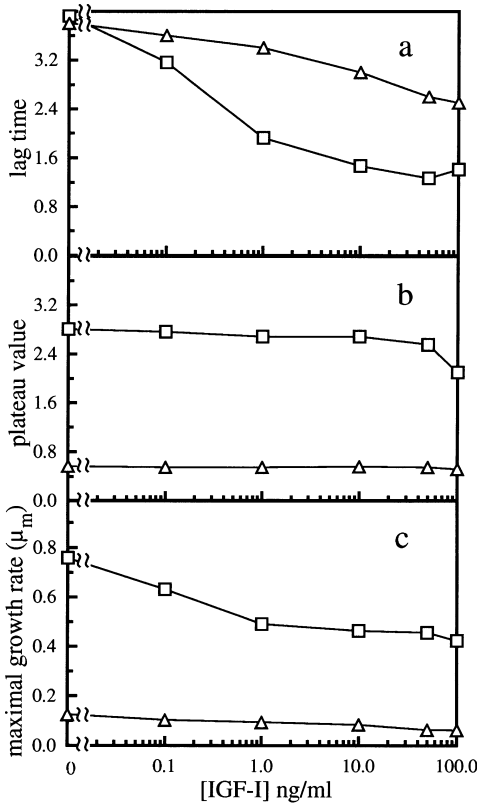

Figure 3 Effects of IGF-I of osteoblast growth parameters

Osteoblasts derived from a 4-year-old (□) and a 90-year-old (△) donor that had been cultured as described in the legend to Figure 2 were used to solve for the growth parameters lag time (**a**), plateau value (**b**) and maximal growth rate (**c**). The growth parameters were calculated by fitting Crystal Violet-binding data (proportional to cell number) to a modified logistic curve as described in the Materials and methods section.

2b) patients. To quantify the observed differences in growth curve profiles, the logistic function was solved for three biologically relevant growth parameters: plateau value (which reflects the maximum cell density upon confluence), the maximal growth rate (μ_m) and lag time. Previous work has shown an age dependence in osteoblast-like strains of both plateau and μ_m values, whereas lag time values were not correlated with age [18]. As might be expected for a competence factor, increasing doses of rhIGF-I decreased the lag time as more cells exited a quiescent stage and entered active proliferation (Figure 3a). The lag time for young osteoblastic cells decreased by 20% at 0.1 ng/ml and by 60% at 10 ng/ml rhIGF-I. The decrease in lag time reached a maximum and stayed constant between 10 and 100 ng/ml rhIGF-I. The magnitude of the decrease in lag times was less for the older osteoblastic cells, with the lag time decreasing by 15% at 0.1 ng/ml and by 45% at 100 ng/ml rhIGF-I. The plateau values for young and old osteoblastic cells exhibited little change with rhIGF-I dose, only changing at the highest dose, 100 ng/ml (Figure 3b). In contrast with the rhIGF-I dose-dependent stimulation of [^{3}H]thymidine incorporation (see above), increasing doses of rhIGF-I decreased μ_m even at the lowest doses for osteoblastic cells (Figure 3c). Osteoblast-like strains derived from 4-year old and 90-year-old donors exhibited a 50% decrease in μ_m at 1.0 ng/ml rhIGF-I and a 100% decrease at 100 ng/ml rhIGF-I.

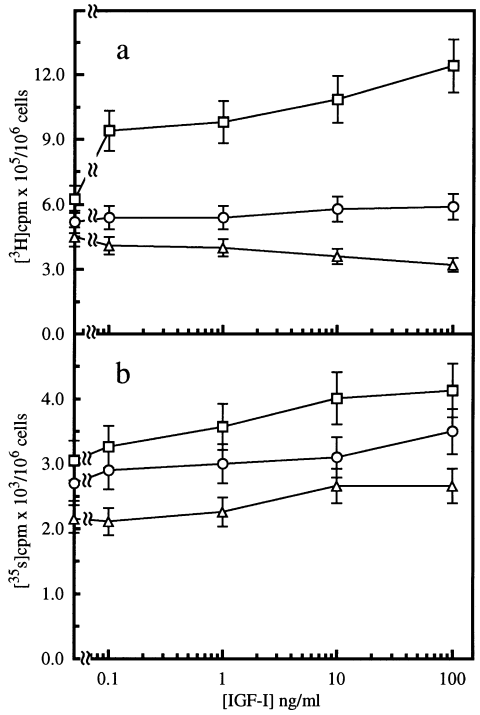

Figure 4 Effects of IGF-I on total protein and proteoglycan levels

Osteoblasts derived from normal bone specimens of three female donors of different ages [14 (□), 40 (○), and 90 (△) years old] were seeded at 50 000 cells/cm^2 and grown for 2 weeks. Cultures were radiolabelled with [^{3}H]proline and [^{35}S]sulphate for 24 h in the presence of various concentrations of rhIGF-I. The incorporation of [^{3}H]proline into total [^{3}H]-labelled protein (**a**) and [^{35}S]sulphate into total [^{35}S]-labelled proteoglycan (**b**) was determined on triplicate wells. Total [^{3}H]-labelled protein and [^{35}S]-labelled proteoglycan radioactivities were normalized on a 10^6 cell basis.

IGF-I and total protein and proteoglycan production

To dissect the effect of rhIGF-I on osteoblast metabolism further, post-proliferative-phase osteoblast-like cells derived from 14-year-old, 40-year-old and 90-year-old healthy female donors were labelled at steady state in the presence of different concentrations of rhIGF-I; the levels of total (medium plus cell layer) protein and proteoglycan were determined as incorporated [^{3}H]proline c.p.m./10^6 cells or [^{35}S]sulphate c.p.m./10^6 cells. Doses of rhIGF-I of 0.1, 1.0, 10 and 100 ng/ml significantly increased ($P < 0.01$) total [^{3}H]-labelled protein by 50%, 60%, 75% and 100% respectively in osteoblast-like cells derived from the 14-year-old donor. Osteoblast-like strains derived from older donors exhibited no significant response to increasing doses of rhIGF-I (Figure 4a). When steady-state levels of total [^{35}S]-labelled proteoglycan were determined as a function of donor age and rhIGF-I concentration, statistically significant differences ($P < 0.05$) were observed only between control and the higher doses (10 and 100 ng/ml) of rhIGF-I in osteoblast-like cells derived from the 14-year-old donor (Figure 4b). The increase in [^{35}S]-labelled proteoglycan levels observed with increasing rhIGF-I dose in the older cell strains was not statistically significant from control values.

IGF-I and extracellular matrix component production

The effect of rhIGF-I on specific osteoblast products was then studied. Confluent osteoblast-like cells derived from a 14-year-old donor were labelled at steady state with [^{3}H]proline (for collagen, thrombospondin and fibronectin levels), [^{35}S]sulphate (for proteoglycan levels) and [^{3}H]glucosamine (for hyaluronan levels) in the presence of various concentrations of rhIGF-I. rhIGF-I increased total [^{3}H]hydroxyproline radioactivity/10^6 cells by 60% at 0.1 ng/ml, 140% at 1 ng/ml, 246% at 10 ng/ml and 200% at 100 ng/ml (Figure 5a). These changes were significant between all doses except the highest (100 ng/ml). The steady-state levels of [^{35}S]biglycan and [^{35}S]decorin also exhibited a dose response (Figure 5b). Total [^{35}S]biglycan increased linearly between 0 and 1.0 ng/ml rhIGF-I and then remained relatively constant at the higher rhIGF-I doses. The 76% increase in [^{35}S]biglycan reached a plateau between 1 and 10 ng/ml rhIGF-I, whereas the 190% increase in [^{35}S]decorin reached a plateau between 10 and 100 ng/ml. Steady-state levels of total [^{3}H]thrombospondin increased by 95% at the lowest dose (0.1 ng/ml) and increased by a further 55% at 1.0 ng/ml rhIGF-I, remaining constant at higher doses (Figure 5c). Levels of total [^{3}H]fibronectin similarly increased 80% at the lowest rhIGF-I dose and increased by an additional 40% at higher doses, although the increases were not significantly different at the higher doses (Figure 5c). In contrast, levels of [^{3}H]hyaluronan and [^{35}S]versican-like proteoglycan decreased by 50% on addition of rhIGF-I (Figure 5d). rhIGF-I did not cause a change in the molecular mass of any of the extracellular matrix components as judged by SDS/PAGE migration (results not shown).

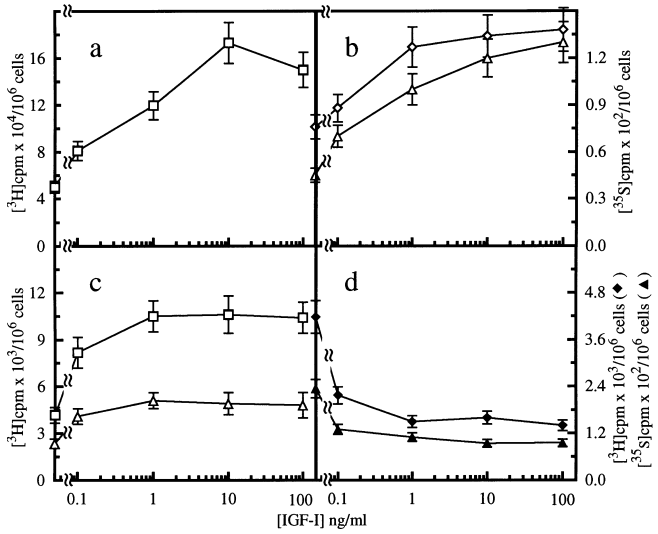

Figure 5 Effects of IGF-I on matrix synthesis

Osteoblasts derived from a 14-year-old healthy female donor were grown for 2 weeks and then radiolabelled at steady state with [^{3}H]proline, [^{3}H]glucosamine and [^{35}S]sulphate in the presence of various concentrations of rhIGF-I. The incorporation of radiolabel into [^{3}H]hydroxyproline was determined as a measure of collagen levels (**a**). The steady-state levels of total (medium plus cell layer) [^{35}S]biglycan (◇) and [^{35}S]decorin (△) (**b**), [^{3}H]thrombospondin (□) and [^{3}H]fibronectin (△) (**c**), as well as [^{3}H]hyaluronan (◆) and [^{35}S]versican-like proteoglycan (▲) (**d**), were determined in triplicate at each rhIGF-I concentration. Matrix component-specific radioactivities were normalized on a 10^6 cell basis.

Age-related effects of IGF-I

The above observations of changes in specific matrix components were in an osteoblast cell strain derived from a 14-year-old. To investigate potential age effects, the responsiveness to rhIGF-I was studied in five additional osteoblast cell strains derived from different age donors. Untreated osteoblasts exhibited an age-dependent change in matrix component production, with

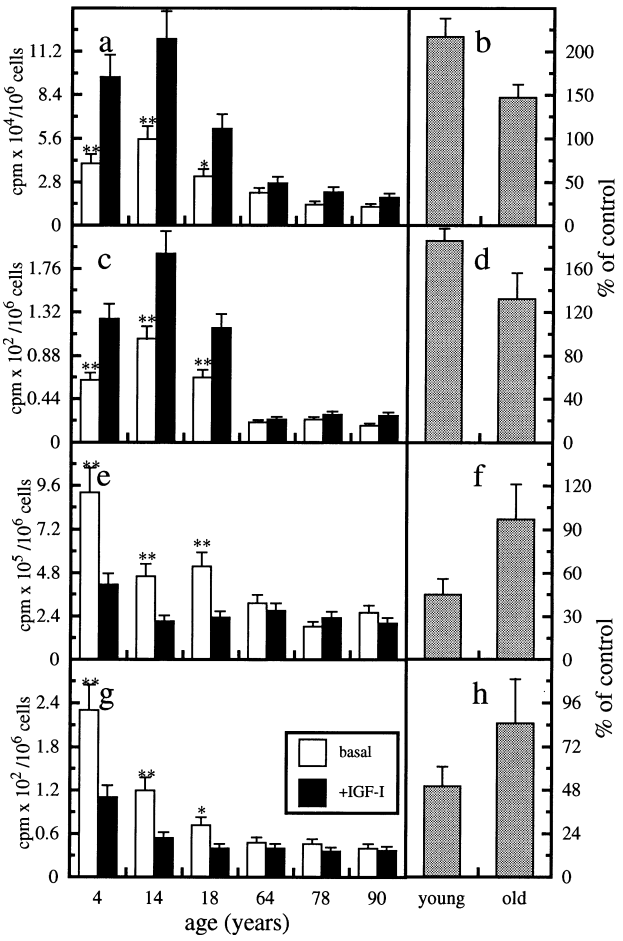

Figure 6 Effects of IGF-I on osteoblasts of different ages

Six osteoblast strains derived from female donors of different ages were grown as described in the legend to Figure 4. They were radiolabelled at steady state with [^{3}H]proline, [^{3}H]glucosamine and [^{35}S]sulphate in the presence (filled bars) or absence (open bars) of 10 ng/ml IGF-I. Label incorporation into [^{3}H]collagen (**a**), [^{35}S]decorin (**c**), [^{3}H]hyaluronan (**e**) and [^{35}S]versican-like proteoglycan (**g**) was determined on triplicate wells as described in the legend to Figure 5. The six strains were grouped as young (ages 4, 14 and 18 years) and old (ages 64, 78 and 90 years) and the results expressed as percentages of control (c.p.m./10^6 cells with IGF-I)/(c.p.m./10^6 cells control). The means and S.D. for collagen (**b**), decorin (**d**), hyaluronan (**f**) and the versican-like proteoglycan (**h**) were determined for both age groups. * $P \leqslant 0.05$, ** $P \leqslant 0.01$, by Student's t test, compared with control.

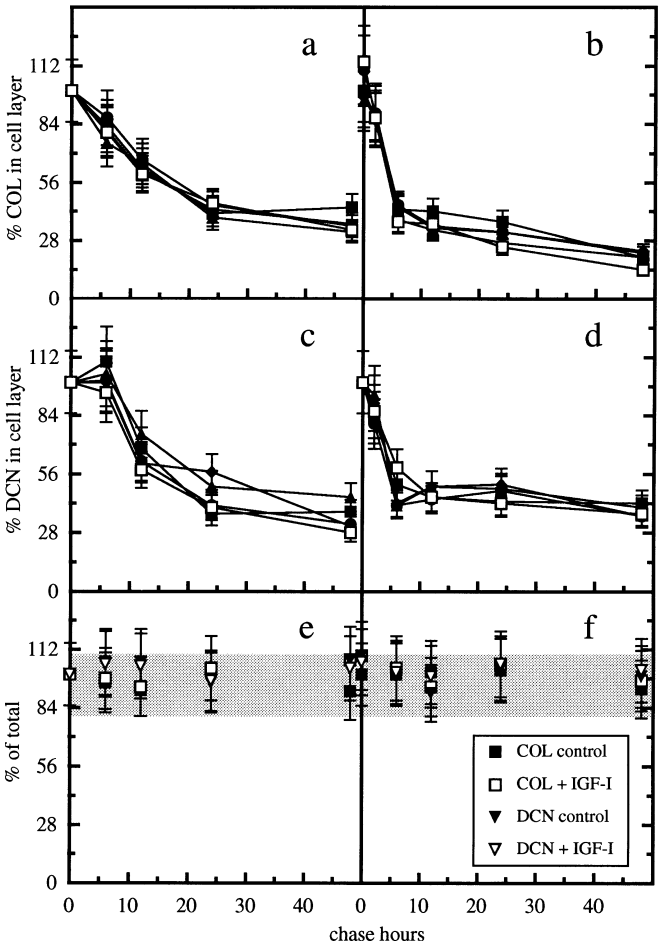

Figure 7 Effects of IGF-I on matrix turnover

Osteoblast-like strains derived from a 14-year-old and a 90-year-old donor were grown for 2 weeks in culture. After a 24 h wash-out period in serum-free, antibiotic-free medium, the cultures were pulsed for 4 h with 100 μCi/ml $H_2{}^{35}SO_4$ and 50 μCi/ml [^{3}H]proline and chased in fresh medium containing 1 mM unlabelled sulphate and proline in the presence of different doses of rhIGF-I. The levels of [^{3}H]collagen and [^{35}S]decorin were determined at times indicated of the chase and the percentage of collagen (**a**, **b**) and decorin (**c**, **d**) remaining in the cell layer pool was calculated for the osteoblast-like cells derived from the 14-year-old (**a**, **c**) and 90-year-old (**b**, **d**) donors. The concentrations of rhIGF-I were the same as those shown in Figure 2. The percentage total (compared with the initial value at the start of the chase) levels of collagen and decorin in control cultures and in the presence of 100 ng/ml rhIGF-I were determined throughout the chase for the osteoblast-like cells derived from the 14-year-old (**e**) and 90-year-old (**f**) donors. The shaded background defines 1 S.D. for the mean of all values. Symbols: ■, collagen control; □, collagen plus IGF-I; ▼, decorin control; ▽, decorin plus IGF-I.

levels of these matrix components decreasing with increasing age (Figure 6). Osteoblast-like cells derived from younger donors exhibited statistically significant responses to rhIGF-I in terms of [^{3}H]collagen, [^{35}S]decorin, [^{3}H]hyaluronan and [^{35}S]versican-like proteoglycan synthesis. Although older osteoblasts did exhibit an rhIGF-I response by an increase in synthesis of collagen and decorin (Figures 6a and 6c) and a decrease in synthesis of hyaluronan and versican-like proteoglycan (Figures 6e and 6g), the magnitude of the change was not statistically significant.

When the values for extracellular matrix components were expressed as a percentage of control (no rhIGF-I treatment) and the strains were grouped into young (ages 4, 14 and 18) and old (ages 64, 78 and 90) populations, the strains derived from younger donors exhibited a greater response to rhIGF-I. Osteoblast-like strains derived from young donors treated with 10 ng/ml rhIGF-I had levels of collagen and decorin (Figures 6b and 6d) that were 200 % of the untreated cohorts. Osteoblast-like strains derived from older donors had collagen and decorin levels that were 140–150 % of control. In young osteoblast-like strains treated with rhIGF-I, hyaluronan and versican-like proteoglycan levels were 50 % of control (Figures 6f and 6h), whereas older osteoblast-like strains had levels that were not statistically significantly different from control.

IGF-I and extracellular matrix turnover

Steady-state radiolabelling produces values that reflect a summation of net synthesis and catabolism. To see whether synthesis or catabolism was being targeted by rhIGF-I, pulse–chase experiments were undertaken to follow collagen and proteoglycan turnover. Treatment of osteoblast-like strains derived from 14-year-old and 90-year-old donors with different doses of

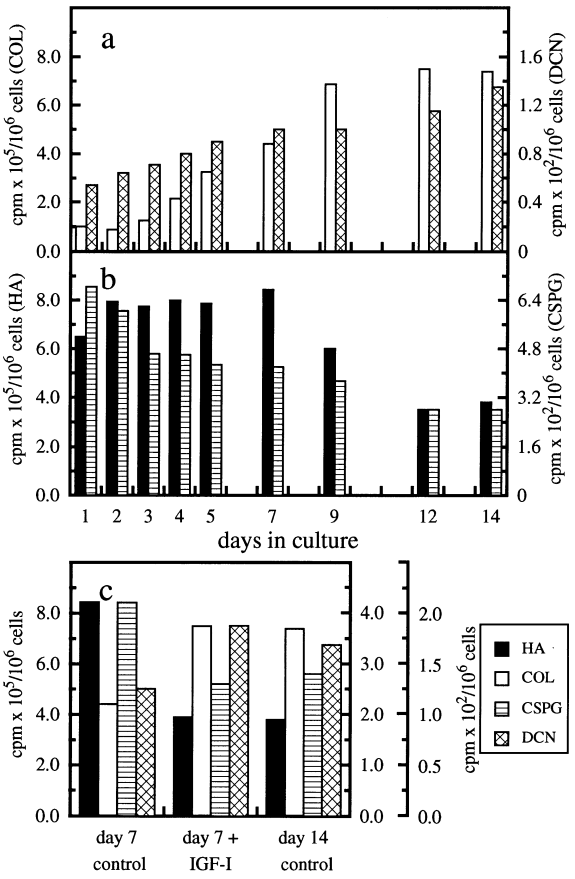

Figure 8 Differentiation *in vitro* and IGF-I

Osteoblasts derived from a 4-year-old female donor were seeded at 20000 cells/cm^2 and labelled at steady state with [^{3}H]proline, [^{3}H]glucosamine and [^{35}S]sulphate on days 1, 2, 3, 4, 5, 7, 9, 12 and 14. Incorporation of radiolabel into collagen (COL) and decorin (DCN) (**a**) and hyaluronan (HA) and versican-like proteoglycan (CSPG) (**b**) was determined as described in the Materials and methods section. On a cohort set of wells, the cells were labelled on day 7 in the presence of 10 ng/ml rhIGF-I. The steady-state levels of collagen, decorin, hyaluronan and versican-like proteoglycan were determined and compared with the levels of these matrix components in control cultures that were not treated with rhIGF-I (**c**).

rhIGF-I was found to have no effect on the stability of bone cell extracellular matrix (Figure 7). Matrix stability was defined by rates of collagen (Figures 7a and 7b) and decorin (Figures 7c and 7d) loss from the cell layer pool for osteoblast-like strains. To determine whether collagen and decorin chased from the cell layer pool were being catabolized, the total (medium plus cell layer) levels of these components were determined throughout the chase. When the levels of total collagen and decorin were expressed as a percentage of the total present at the start of the chase, neither control nor high doses of rhIGF-I exhibited catabolism (Figures 7e and 7f).

IGF-I and osteoblast differentiation

The observations of rhIGF-I treatment's being associated with a decreased μ_m in proliferating osteoblasts and an increase in the synthesis of collagen, decorin and to a smaller degree biglycan, fibronectin and thrombospondin levels in non-proliferating, confluent osteoblast-like cells suggested that rhIGF-I might exert its effect on bone by changing the differentiated state of the cells. To test this hypothesis, steady-state levels of collagen, a versican-like proteoglycan, decorin and hyaluronan produced by osteoblast-like cells were determined throughout 14 days of culture (Figure 8). On day 7 of culture, parallel cultures were treated with 10 ng/ml rhIGF-I during steady-state labelling and the levels of matrix components synthesized were compared with those of untreated cells. When the total (medium plus cell layer) levels of matrix components were analysed during 2 weeks of culture in the absence of rhIGF-I, collagen and decorin (Figure 8a) were induced with length of time in culture, whereas versican-like proteoglycan and hyaluronan (Figure 8b) exhibited high levels early on and a decrease in levels with increasing time in culture. Against this backdrop of a temporal expression pattern *in vitro*, the osteoblast-like cells treated on day 7 of culture with rhIGF-I produced steady-state levels of collagen, decorin, versican-like proteoglycan and hyaluronan that were equivalent to levels of these matrix components produced by control cells on day 14 of culture (Figure 8c).

DISCUSSION

IGF-I has been shown *in vivo* to stimulate bone formation [1,2,7]. Studies have shown that rhIGF-I increased fetal rat metatarsal growth in 18-day-old and 19-day-old organ culture, whereas a response was lacking in a culture derived from a 2-day-old [33]. rhIGF-I stimulated 21-day fetal rat calvaria bone matrix apposition [34]. In parietal bone culture, treatment with IGF-I for 72 h increased calcification, cell processes and osteocytes per unit area of bone [35]. Because osteoblasts produce the osteoid that ultimately becomes mineralized bone, a relevant assay *in vitro* for IGF-I could be extracellular matrix (*in vitro* osteoid) formation. Our initial study of total protein and proteoglycan steady-state levels revealed a modest response to rhIGF-I by osteoblast-like cells *in vitro*. Steady-state levels of collagen, decorin and biglycan were significantly stimulated and thrombospondin and fibronectin were moderately stimulated, whereas hyaluronan and a versican-like proteoglycan were inhibited by IGF-I.

Decorin has been found associated with collagen fibrils [36] and shown to regulate fibrillogenesis *in vitro* [37]. Biglycan [38], thrombospondin [39] and fibronectin [40] have also been shown to bind type I collagen and modulate aggregate stuctures. Previous work has shown that hyaluronan and versican-like proteoglycan levels are highest in osteoblast-like strains derived from fetal and neonatal donors, whereas collagen and decorin levels are highest in strains derived from peri-pubertal donors [19]. In the present study, treatment of osteoblast-like cell strains with IGF-I altered the pattern of expression where markers of mature bone matrix (collagen and decorin) were increased and markers of fetal/neonatal bone matrix (hyaluronan and the versican-like proteoglycan) were decreased. These observations are consistent with IGF-I's promoting osteoblast differentiation.

IGF-I has been shown to stimulate proliferation in numerous osteoblast-like model systems [10–17]. The present studies present an apparent paradox in the effect of rhIGF-I on stimulating DNA synthesis and decreasing lag time while slowing the maximal growth rate attained by an osteoblast strain and altering extracellular matrix expression. The results are consistent with rhIGF-I's promoting the transit of more bone cells from G_0 to G_1 (as a competence factor such as IGF-I is known to do). The decrease in maximal growth rates could be secondary to an increase in the time that it takes cells to transit the cell cycle. Thus osteoblastic cells that are stimulated to enter the cell cycle divide at a slower rate. It is also possible that rhIGF-I affects a specific subpopulation of osteoblasts in the heterogeneous

system *in vitro*. During the course (14 days) of treatment with rhIGF-I, that subpopulation with a distinct proliferation rate is selected by rhIGF-I, becomes the dominant clonal population and alters maximal growth rate. Whatever the mechanism, the decreased growth rate in the presence of IGF-I is associated with altered production of osteoblast extracellular matrix. In non-proliferating osteoblasts, rhIGF-I altered the production of extracellular matrix components so that the pattern of matrix component expression was equivalent to that of more differentiated osteoblasts (where differentiation is defined by temporal changes in matrix synthesis and elaboration). Given that, *in vivo*, IGF-I increases bone apposition and that the osteoblasts are not usually proliferating, the more physiologically relevant effect of IGF-I *in vitro* might be the alteration of the synthesis of specific extracellular matrix compartments.

A role for IGF-I in regulating bone matrix turnover has yet to be clearly defined. The role of rhIGF-I as a physiological peptide that regulates extracellular matrix homoeostasis in cartilage by increasing anabolism and decreasing catabolism has been well established [41–47]. IGF-I has been found to affect the extracellular matrix in osteoblast-like cells by decreasing interstitial collagenase transcripts as well as induced protease levels in osteoblast cells, thereby regulating the degradation of bone collagen [48]. The current results indicate that rhIGF-I, during the time frame of the pulse–chase employed, did not affect catabolism rates for proteoglycan or collagen in human osteoblast-like cells. Osteoblasts are further different from chondrocytes in that in chondrocytes IGF-I did not affect biglycan or decorin levels [49].

So far, only a few studies have been published characterizing human osteoblast-like cell responsiveness to hormones and growth factors as a function of age [21,50,51]. Cells from 61–70-year-old donors required approx. 10-fold higher concentrations of growth factors and hormones to yield comparable increases in DNA synthesis than cells from 51–60-year-old donors [21]. A linear decline in the skeletal content of IGF-I has been found *in vivo* with increasing donor age [52]. Thus osteoblasts *in vivo* might be presented with a diminishing IGF-I stimulus in older individuals. In the present system *in vitro*, osteoblast-like cells derived from older donors exhibited a diminished response to IGF-I. These results parallel the generalized decrease in adrenergic responsivity with aging observed in multiple cells and tissue types [53–59]. The decrease in adrenergic response is associated with dysfunction of the receptor signal-transducing pathway [60,61].

The age-dependent decrease in human osteoblastic responsiveness to rhIGF-I observed in the present study could be explained by three different hypotheses. The first potential hypothesis is that the age-related decreases are part of a generalized decrease in cellular metabolism associated with aging. If this were the case, osteoblast-like cells derived from older donors would never show the magnitude of response to IGF-I that 'younger' cells exhibit. The second possibility is that the age-related decreases result from changes in ligand–receptor signalling. That is, with increasing age of donor, the signal propagated by IGF-I binding to its receptor is of a smaller magnitude. Finally, it is possible that the age-related decreases are artifacts of the methods employed: that is, treatment with IGF-I for 24 h and determining steady-state levels of extracellular matrix proteins in an osteoblast-like strain derived from an 18-year-old is not equivalent to a 24 h treatment of a cell strain derived from a 72-year-old. The osteoblast-like cells derived from older donors require a different time frame of treatment and analysis. If this were the cause, 'older' osteoblasts would eventually yield the same magnitude of response as 'younger' osteoblasts if the experimental conditions were changed. Further work will distinguish between these potential hypotheses.

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