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Bone Loss (Osteopenia) in Old Male Mice Results From Diminished Activity and Availability of TGF-β

Dan Gazit,[1] Yoram Zilberman,[1] Reinhard Ebner,[2] and Arnold Kahn,[3]*

[1]Molecular Pathology Laboratory, Hebrew University-Hadassah Faculty of Dental Medicine, Jerusalem, Israel
[2]Human Genome Sciences, Rockville, Maryland
[3]Department of Growth and Development, UCSF School of Dentistry, San Francisco, California

Abstract One of the universal characteristics of the long bones and spines of middle-age and older mammals is a loss in bone mass (osteopenia). In humans, if this bone loss is severe enough, it results in osteoporosis, a skeletal disorder characterized by a markedly increased incidence of fractures with sequelae that may include pain, loss of mobility, and in the event of hip fracture, even death within a relatively few months of injury. An important contributing factor to the development of osteopororsis appears to be a diminution in the number and activity of osteoblasts responsible for synthesizing new bone matrix. The findings in the present and other similar studies suggest that this reduction in osteoblast number and activity is due to an age-related diminution in the size and osteogenic potential of the bone marrow osteoblast progenitor cell (OPC or CFU-f) compartment. We previously postulated that these regressive changes in the OPC/CFU-f compartment occurred in old animals because of a reduction in the amount and/or activity of TGF-β1, an autocrine growth factor important in the promotion of OPC/CFU-f proliferation and differentiation. In support of this hypothesis, we now report that (1) the osteogenic capacity of the bone marrow of 24-month-old BALB/c mice, as assessed in vivo, is markedly reduced relative to that of 3–4-month-old animals, (2) that the matrix of the long bones of old mice contains significantly less TGF-β than that of young mice, (3) that OPC's/CFU-f's isolated from old mice produce less TGF-β in vitro than those recovered from young mice, and (4) that OPC's/CFU-f's from old mice express significantly more TGF-β receptor (Types I, II, and III) than those of young animals and that such cells are more responsive in vitro to exogenous recombinant TGF-β1. We also find that colony number and proliferative activity of OPC's/CFU-f's of young mice and old mice, respectively, are significantly reduced when incubated in the presence of neutralizing TGF-β1 antibody. Collectively, these data are consistent with the hypothesis that in old male mice the reduction in the synthesis and, perhaps, availability from the bone matrix of TGF-β1 contributes to a diminution in the size and development potential of the bone marrow osteoprogenitor pool. J. Cell. Biochem. 70:478–488.
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TGF-β, one the most abundant growth factors in the bone matrix, acts on mesenchymal cells as a powerful stimulator of cell proliferation and extracellular matrix production [Roberts et al., 1988; Hock et al., 1990; Dallas et al., 1995]. Although its precise role in skeletal development and modeling remains uncertain, some of the available data suggest that it is an important, perhaps essential, component in the autocrine/paracrine cascade leading to osteoblastic differentiation [Bonewald et al., 1994; Centrella et al., 1994; Erlebacher et al., 1995; Aspenberg et al., 1996; Erlebacher et al., 1996]. However, this "anabolic" effect is dependent on the stage of maturation of the differentiating cells and length of exposure to the growth factor. For example, continuous exposure of immature cells to TGF-β may inhibit rather than stimulate osteoblastic differentiation [Centrella et al., 1994; Aspenberg et al., 1996; Erlebacher et al., 1996]. Within this context, it is of interest that in some model systems of osteogenesis, endogenous expression of TGF-β1 is an early event in

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*Correspondence to: Arnold Kahn, Department of Growth and Development, UCSF Medical Center, 521 Parnassus Ave., San Francisco, CA 94143–0640.

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the developmental sequence and may be followed by downregulation in TGF-β receptor expression and lower levels of growth factor activity [Joyce et al., 1990; Bolander, 1992; Gazit et al., 1993]. In either case, the long-term, potentially inhibitory action of this growth factor would be reduced [Rosen et al., 1994].

One of the consequences of the aging process beyond middle age and the reproductive period is a loss in bone mass and load-sustaining bone architecture [Kahn et al., 1995; Bergman et al., 1996]. When these losses extend beyond the ability of the tissues to sustain loads, they lead to an increased incidence of bone fracture and the sequelae that sometimes follow such injuries. This condition, historically known as "senile" osteoporosis (Type II osteoporosis), occurs in both elderly women and men. Although the mechanisms responsible for Type II osteoporosis are not fully understood, an important component appears to be a decline in the numbers and, perhaps, function of the osteoblasts that are responsible for synthesizing new bone matrix during skeletal remodeling [Nicolas et al., 1994; Manolagas et al., 1995; Parfitt et al., 1995; Quarto et al. 1995]. Under these conditions, even in the absence of an increase in the leel of bone resorption, a loss in bone mass will occur [Manolagas and Jilka, 1995].

The osteoblasts that line the endosteum and the surfaces of trabecular bone appear to be derived from multipotential stem cells located in the bone marrow stroma [Kahn et al., 1995; Zhang et al., 1995; Berman et al., 1996; Simmons, 1996]. These stem or progenitor cells can be grown in tissue culture, are clonogenic (i.e., give rise to discrete colonies in vitro) and can be osteogenic in the absence of evident exogenous inductive stimulation. Typically, such cells are referred to as CFU-f (colony forming unit-fibroblast), OPCs (osteoprogenitor cells), or MSCs (mesenchymal stem cells) [Owen, 1988; Owen and Freidenstein, 1988; Kahn et al., 1995; Bruder et al., 1997; Jaiswal et al., 1997; Krebsach et al., 1997; Prockop, 1997]. Cumulative, previously reported observations strongly suggest that the number and osteogenic potential of marrow CFU-f declines with advancing age. If this is so, it would provide a partial explanation for the reduction in osteoblast number and a lessening in bone forming capacity in old animals, including humans [Tsuji et al., 1990; Kahn et al., 1995; Inoue et al., 1997].

In an earlier preliminary study, we reported evidence for an age-related loss in CFU-f in the marrow of old male mice and postulated that this loss might be accounted for by an age-related reduction in the level or activity of the TGF-β needed to promote the proliferation of the CFU-f and their differentiation into osteoblasts [Kahn et al., 1995]. We have now developed additional evidence for an age-related decline in the CFU-f population as well as experimental findings that support the notion that TGF-β diminishes in level and activity with age. Some of these data, derived largely but not exclusively from in vitro analysis, are presented below. Other results, which are based upon experiments on old mice treated with recombinant TGF-β1 will be reported separately (Gazit et al., unpublished communications). Collectively, these observations show that less TGF-β is produced by marrow osteogenic cells and stored in the matrix of long bones in old male BALB/c mice, that the CFU-f of old animals are more responsive to exogenous TGF-β1 than the CFU-f of young mice, and that bone formation in old mice is selectively stimulated (relative to young animals) by injections of TGF-β1.

MATERIALS AND METHODS

Tissue Culture Supplies and Mice

Recombinant human TGF-β1 and neutralizing antibody to TGF-β1 were obtained from R&D Systems (Minneapolis, MN). Fetal bovine serum, α-MEM tissue culture medium, and other tissue culture supplements were purchased from Bet Haemek (Israel). All other reagents were purchased from Sigma (St. Louis, MO). BALB/c mice were obtained from Charles River , in part, through a contractual arrangement with the National Institute of Aging.

Bone Marrow Transplantation and Related Histology

Bone marrow was isolated from the femurs of 3- and 24-month-old donor male BALB/c mice and transplanted under the kidney capsule of 3-month-old male BALB/c recipients. The femurs of donor animals were recovered using aseptic technique, their epiphyses removed, and the contents of the marrow cavity expelled under hydrostatic pressure using cold tissue culture medium delivered into the marrow space with a 22G needle syringe. A small tear was made in the renal capsule of an anes-

thetized host mouse (2% Xylazine and 8.5% Ketamine, i.p.), and the bone marrow from each femur placed beneath the kidney capsule with the aid of a spatula. After 3 weeks, the transplants and associated kidney tissue were recovered, fixed in 4% formaldehyde, and decalcified with De-Cal Rapid (National Diagnostics, Atlanta, GA) overnight at room temperature. The tissues were then embedded in paraffin using standard techniques and 5 um sections cut and mounted on slides. The sections were stained with Masson Trichrome. Five separate marrow transplants from young and old mice donors were analyzed and gave similar results.

Cell Culture

Modifications of previously described techniques were used to culture stromal CFU-f [Tsuji et al., 1990; Leboy et al., 1991; Beresford et al., 1994; Cheng et al., 1994; Richard et al., 1994; Inoue et al., 1997] . In brief, bone marrow was expelled from the tibial and femoral diaphyses as described above. The marrow cells were resuspended gently in medium by passage through a series of 19 G, 21 G, and 23 G needles, counted and, after appropriate dilution, seeded into 60 mm plastic culture dishes (Nunc, Denmark) at a density of 4 x 10^6 cells/60 mm plate. The cells were then cultured at 37°C in α-MEM medium supplemented with 100 u/ml of Pen-Strep and 10% FBS. The medium was changed after 48 h of culture and twice a week thereafter. Ten mM β-glycerophosphate, 10^{-8} M dexamethasone, and 50 mg/ml ascorbic acid were added to the medium with the first change of media. Typically, CFU-f cultures were assayed after 7 days of incubation. However, for TGF-β1 binding assays, secondary as well as primary CFU-f cultures were used. Secondary cultures were prepared by trypsinizing and then subculturing 12 day CFU-f cultures (10^6 cells) into 6 well plastic cultures dishes (35 mm diameter wells; Nunc) for an additional 12 days.

Recombinant TGF-β1 and Anti-B1 Antibody In Vitro

CFU-f were cultured for the first 48 h in medium supplemented with 20% FCS and from days 2–7 under reduced 0.5% (FBS) serum conditions. The cells were treated on days 0, 2, and 5 with 1 ng/ml or 10 ng/ml TGF-β1 or 1,000x amount (w/w) of neutralizing, anti-TGF-β1 antibody [Van Vlasseaer et al., 1994; Hughes et al., 1996]. On day 7, the cultures were fixed and stained for alkaline phosphatase activity (ALP) as described below.

Alkaline Phosphatase Histochemistry, Colony Count, and Histomorphometry

On day 7 of culture, six 60 mm plates of both donor age groups were washed with PBS, fixed with citrate-acetone-formalin, and stained for alkaline phosphatase activity (Sigma 86-R kit). Total colonies and alkaline phosphatase positive colonies (>50% positive staining cells) were counted, a colony being a group or cluster containing 16 or more cells. The scores from each of the six well plates were averaged for each age group. CFU-f size was determined using a computerized morphometric system (Galai: CUE-3 Electro Optical Inspection and Diagnostic Laboratories, Migdal Haemek, Israel).

Determination of TGF-β1 Secreted Protein Level

CFU-f were obtained from 4 young and 4 old mice as described above. TGF-β1 concentrations in the conditioned medium were quantified using a TGF-β1-specific ELISA kit (R&D Systems) according to the manufacturer's instructions. All samples were assayed in triplicate. Samples were acid-activated [Lawrence et al., 1985] by the addition of 1/10th vol. of 1.1N HCl at room temperature and neutralized after 20 min by the addition of 1/5 vol. of 0.72 N NaOH in O.5 M Hepes. The cells were then lysed in double distilled water (DDW), the residue scraped from the plate surfaces, and total protein measured according to Bradford [1976].

TGF-β Content in Bone Matrix

Bone extracts were prepared from 3- and 24-month-old mice, respectively, as previously described [Finkelman et al., 1992]. Femurs, tibias, and lumbar vertebrae were cleaned of soft tissue and the epiphyses and bone marrow removed. The bones were crushed and the pieces washed, successively, in double-distilled water (20 min), 95% ethanol, 100% ethanol (20 min each),, and chloroform/methanol (1:1; 1 h). The bone fragments were dried overnight at 30°C, pulverized (particle size, 90–850 mm^2), and frozen at −70°C until extracted. For the latter purpose, the particles were washed for 1 h in 4 M guanidine hydrochloride (Gdn.HCl)/30 mM Tris HCl, (pH 7.4) containing protease inhibitors (5 mM benzamidine, 100 mM e-aminoca-

proic acid, and 1 mM phenylmethylsulfonyl fluoride) to remove nonmineral-bound proteins. Bone particles were then extracted by dialysis (Spectra/Pore 3; MW cutoff, 3,500; Spectrum Medical Industries) against 4 M Gdn HCl/30 mM Tris HCl, pH 7.4, containing 10% (wt/vol.) Na4EDTA and protease inhibitors, the dialysate desalted and clarified by centrifugation. The Gdn HCL/EDTA supernatants were then lyophilized. TGF-β levels in residue was determined in a bioassay measuring the inhibition of DNA synthesis in mink lung epithelial cells (Mv 1 Lu:CCL 64, American Type Culture Collection) [Jennings et al., 1988]. Purified TGF-β1 protein from porcine platelets (R&D Systems was used as the standard).

^{125}I-TGF-β1 Binding and Receptor Number

The medium of primary and secondary CFU-f cultures from young and old mice were replaced on day 12 with serum-free medium 24 h prior to determination of TGF-β1 surface-binding capacity. The culture wells were washed thoroughly with binding buffer (Krebs-Ringer containing 20 mM Hepes and 0.5% BSA) and incubated with 50 pM ^{125}I-TGF-β1 9 (Amersham Corp., Buckinghamshire, UK, 106 uCi/ug] in binding buffer for 4 h at 4°C to reach equilibrium conditions. After removing unbound ligand from the binding medium, the bound tracer was chemically crosslinked to the cells with 0.15 mM disuccinimidyl suberate [Wang et al., 1991]. The cells were then recovered by scraping into 1 ml binding buffer and centrifuged. The resulting pellets were resuspended in 60 ul of lysis buffer (10 mM Tris-HCl, pH 7.4, 1 ml EDTA, pH 8.0, 1% Triton X-100 with 5 ug/ml aprotinin, 5 ug/ml leupeptin, and 1 ml PMSF). After centrifugation, the soluble fraction was mixed with 5X Laemmli sample buffer containing 25% β-mercaptoethanol and separated on a 12.5% SDS-poly-acrylamide gel. After fixation in 50% methanol/10% acetic acid, the gel was autoradiographed (X-Omat Kodak R film) using DuPont LightningPlus intensifying screens. The molecular weight markers were ^{14}C-labeled myosin (H- chain; 200 kDa), phosphorylase-b (97.4 kDa), BSA (68 kDa), and ovalbumin (43kDa). The relative intensities of the bands were quantified by scanning laser densitometry (computing densitometer 300E, Molecular Dynamics, Eugene, OR; Imagequant software).

Statistical Analysis

Quantitative data were analyzed using the Mann Whitney-U test [Siegel, 1956] and are presented in the text as the mean +/-SEM. All experiments were performed in triplicate; however, only representative results are shown.

RESULTS

Bone Marrow Osteogenic Potential Is Reduced in Old Mice

Our previous findings of reduced OPC/CFU-f level in the bone marrow of old mice [Kahn et al., 1995] are indirectly but independently confirmed in the present in vivo experiments involving the transplantation of bone marrow cells from old and young animals to the renal capsules of young recipients. Because the marrow of young animals contains larger numbers of OPCs as assessed in vitro [Kahn et al., 1995] it might be expected to generate a larger amount of bone at the graft site. As can be seen in Figure 1A,B, the bone ossicles formed from young animal marrow grafts are indeed substantially larger than those observed in marrow transplants from old mice. In addition, bone marrow developed only in association with osteogenic tissue derived from young mice. Ossicles from both aged donors were generally located at the same distance beneath the kidney capsule and were surrounding by the same types of cell.

OPCs/CFU's of Old Mice Produce Lower Levels of TGF-β In Vitro and Apparently In Vivo

As noted in the beginning, the marrow CFU-f (OPCs) of old mice are reduced in number, alkaline phosphatase activity, and bone-forming activity [Kahn et al., 1995]. Similarly, when TGF-β1 production is measured in such cells in vitro, it is reduced by a factor of ~2 in comparison to that synthesized by CFU-f from young animals (Fig. 2). This lower growth factor production parallels and is probably partly responsible for the markedly reduced (~16 x) TGF-β levels observed in the bone matrix of the tibias and femurs of old mice (Fig. 3). Interestingly, and for reasons not yet understood, the reduction in matrix growth factor content seen in the long bones of old mice is not evident in the spines of the same animals (Fig 3).

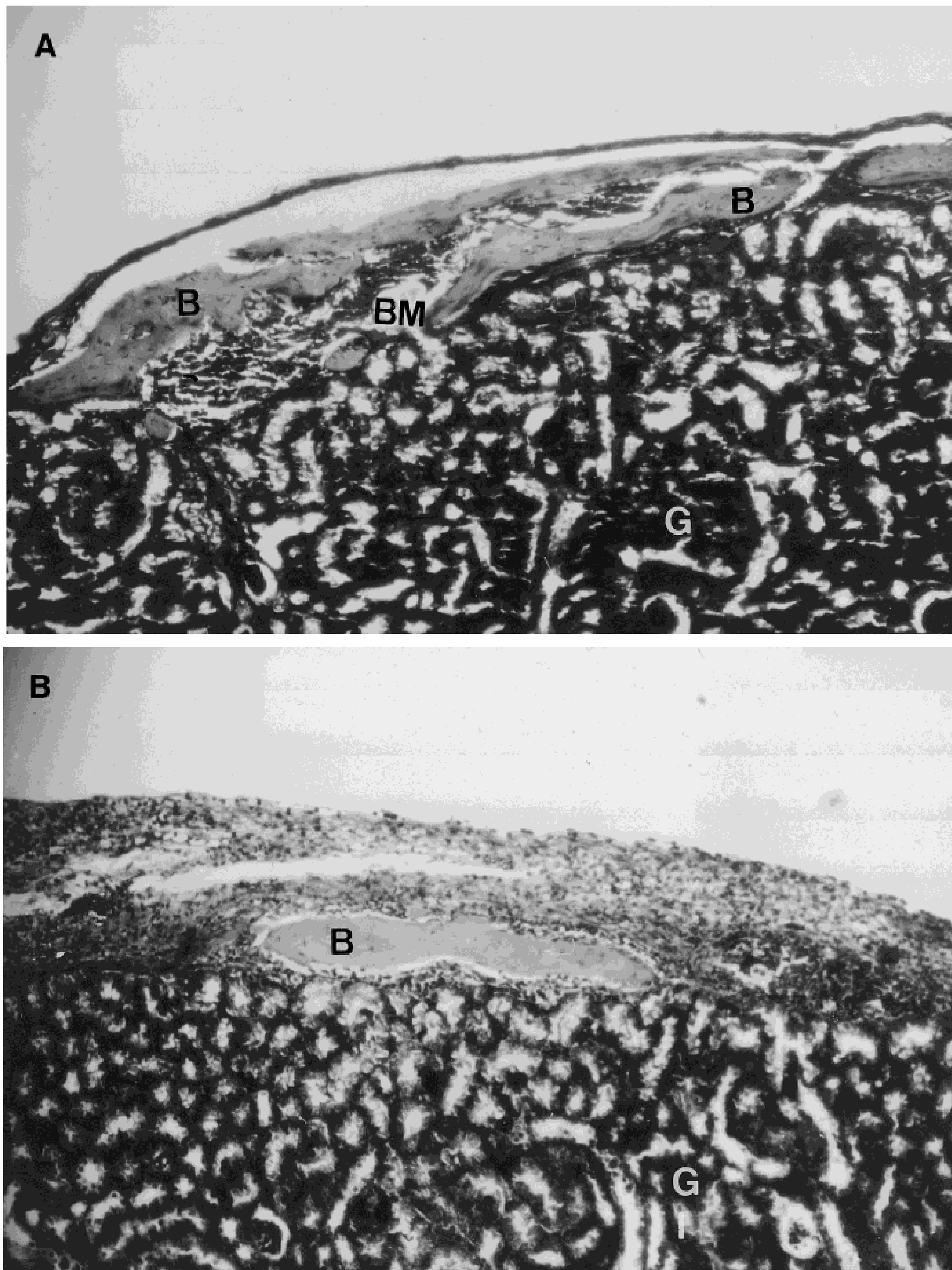


Fig. 1. Bony ossicles (B) formed in 3-month-old mice following transplantation of marrow from young (**A**) and old (**B**) mouse femurs. Note the smaller amount of bone formed in response to 24-month-old donor marrow (B) relative to that seen following the introduction of 4-month-old donor cells (A). Also note that bone marrow (BM) formation is associated only with the ossicle formed from young donor cells (A). These results are consistent with a reduced osteogenic potential of the bone marrow from old mice. Masson Trichrome stain, original magnification $\times 25$.

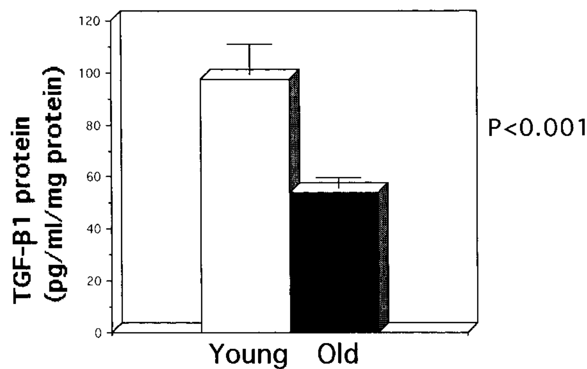


Fig. 2. Histogram representing the amount of TGF-β1 protein present in the conditioned medium of CFU-f cultures started from young and old mice. Note that the CFU-f from the 24-month-old mice secrete significantly less protein as determined by ELISA than the CFU-f from 4-month-old animals. These results represent the mean ± SE in triplicate cultures from three experiments.

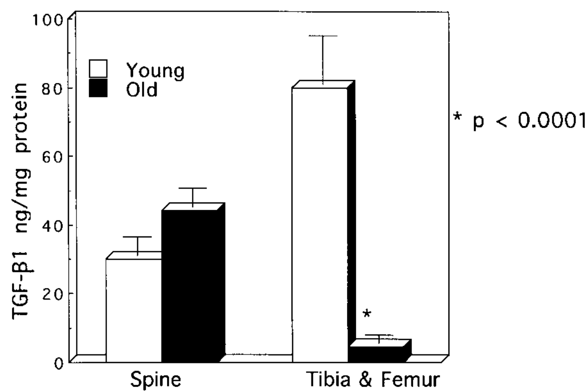


Fig. 3. TGF-β1 levels in the bone matrix of young and old mice. TGF-β was extracted with guanidinium and bioassayed using Mink Lung Cells as previously described. Note that the level of TGF-β protein in the tibiae and femurs is significantly reduced in the 24-month-old animals. The results represent the mean ± SE of tissue from 10 animals in one large experiment.

CFU-f/OPC From Old Mice Exhibit Elevated Expression of TGF-β Receptor and Greater Response to TGF-β1 In Vitro

Expression of TGF-β receptors is sharply elevated in old mouse CFU-f as measured in cross-linking receptor assays using radiolabeled TGF-β1 (Fig. 4A,B). Lack of differences in Scatchard analysis from binding experiments and displacement kinetics in competition studies indicate that the observed increase in TGF-β binding is predominantly due to a change in the number of binding sites, rather than to a shift in binding affinities (data not shown). This difference in number is true for all three major types of TGF-β receptor but is most striking for receptor types I and II. In all cases, receptor abundance in the CFU-f from old animals is ~10- to 20-fold greater than in that of cells from young animals. Moreover, the difference persists even after the cells are passaged a second time (Fig. 4A,B).

Given this discrepancy in receptor number, it is not surprising that CFU-f cultured from old animals are more sensitive to recombinant Human TGF-β1 (rTGF-β1) than CFU-f derived from young mice (Fig. 5A,B). As can be seen in Figure 5A, the addition of either 1 or 10 ng/ml of the growth factor to cultures of marrow cells from old mice significantly increases the number of ALP(+) colonies, whereas only the higher dosage is effective in similar cultures of marrow cells derived from young animals. These data reflect colony formation from day 0 of marrow culture and, therefore, can be correlated with the initial number of progenitors that adhere and initiate proliferation. Moreover, the degree of response, i.e., percent increase in number of colonies, is much greater in cultures of old mouse marrow cells (Fig. 5A).

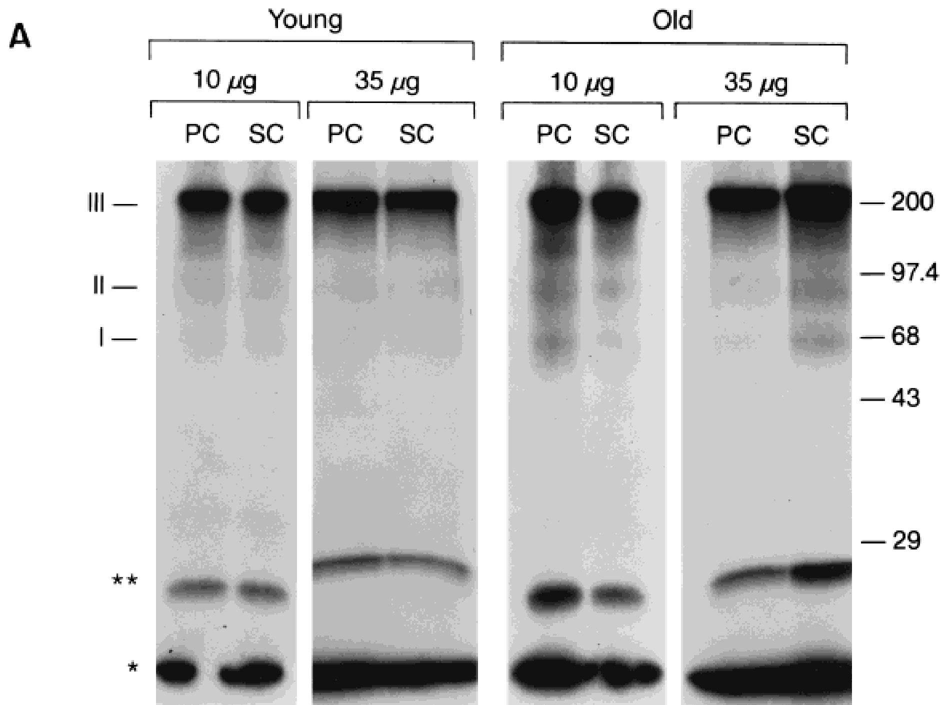
A similar difference in responsiveness to rTGF-β1 is also seen when colony size (which presumably reflects a higher in vitro proliferation rate of the osteoprogenitors) is measured in cultures containing the higher level of growth factor, 10 ng/ml. As can be seen in Figure 5B, TGF-β1 elicits an approximately sixfold greater increase in colony size in the CFU-f derived from old animals as opposed to a ~fourfold gain in young mouse CFU-f colonies.

Endogenously Produced TGF-β Influences CFU-f Colony Size and Number In Vitro

When neutralizing antibody to TGF-β1 is added to marrow cell cultures, the baseline levels of colony formation and proliferative activity are reduced (Fig. 5A,B). In CFU-f cultures from young animals, the presence of antibody reduces colony number and size by ~20% (Fig. 5A) and ~30% (Fig. 5B), respectively. However, only the reduction in colony number reaches statistical significance. In contrast, the addition of antibody to cultures established from old mouse marrow, produces no change in colony number but does significantly reduce colony size by ~70% (Fig. 5B).

DISCUSSION

One of the universal characteristics of the long bones and spines of aged animals is the



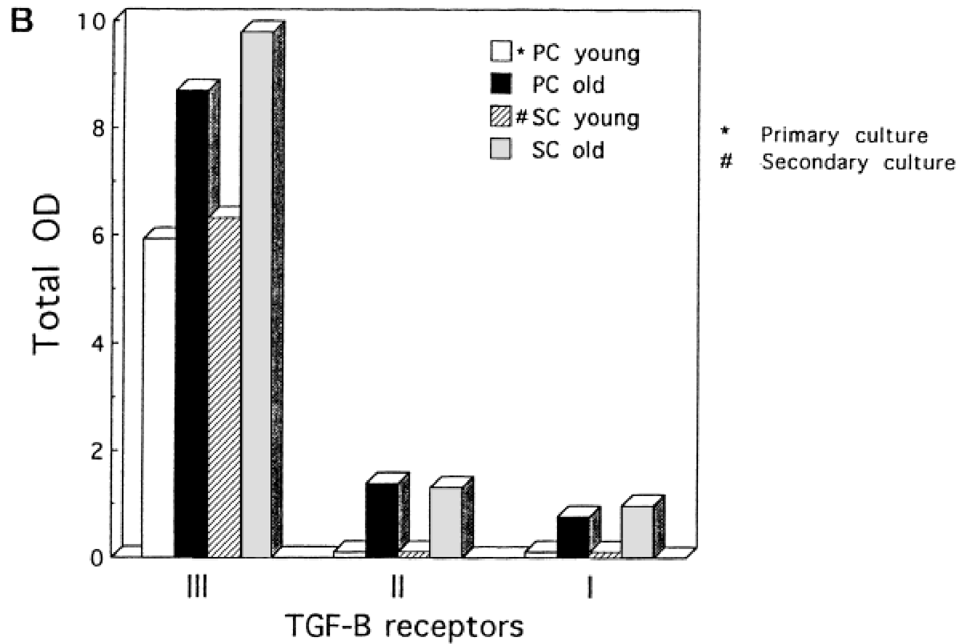


Fig. 4. TGF-β1 receptor levels (types I, II, and III) in primary (PC) and secondary (SC) bone marrow CFU-f cultures prepared from young and old mice. **A.** After binding of 50 pM ^{125}I TGF-β (106 uCi/ug) to the intact cells, the radiolabeled ligand was cross-linked and the radiolabeled ligand-receptor complexes electrophoretically separated in denaturing polyacrylamide gels. Nonspecific binding was determined in the presence of a 200-fold molar excess of unlabeled TGF-β. Note the increased binding of ^{125}I-TGF-β1 to receptors in cultures obtained from old animals. **B.** Densitometric histogram representing the relative intensity of the receptor bands in the autoradiogram shown in A. The three receptor types (I, II, and III) are marked with Roman numerals. Noncrosslinked dimeric tracer (~25 kd) is indicated by single and double asterisks. The positions of the molecular weight markers are indicated. The data presented are from one representative experiment of three independent experiments that gave identical patterns.

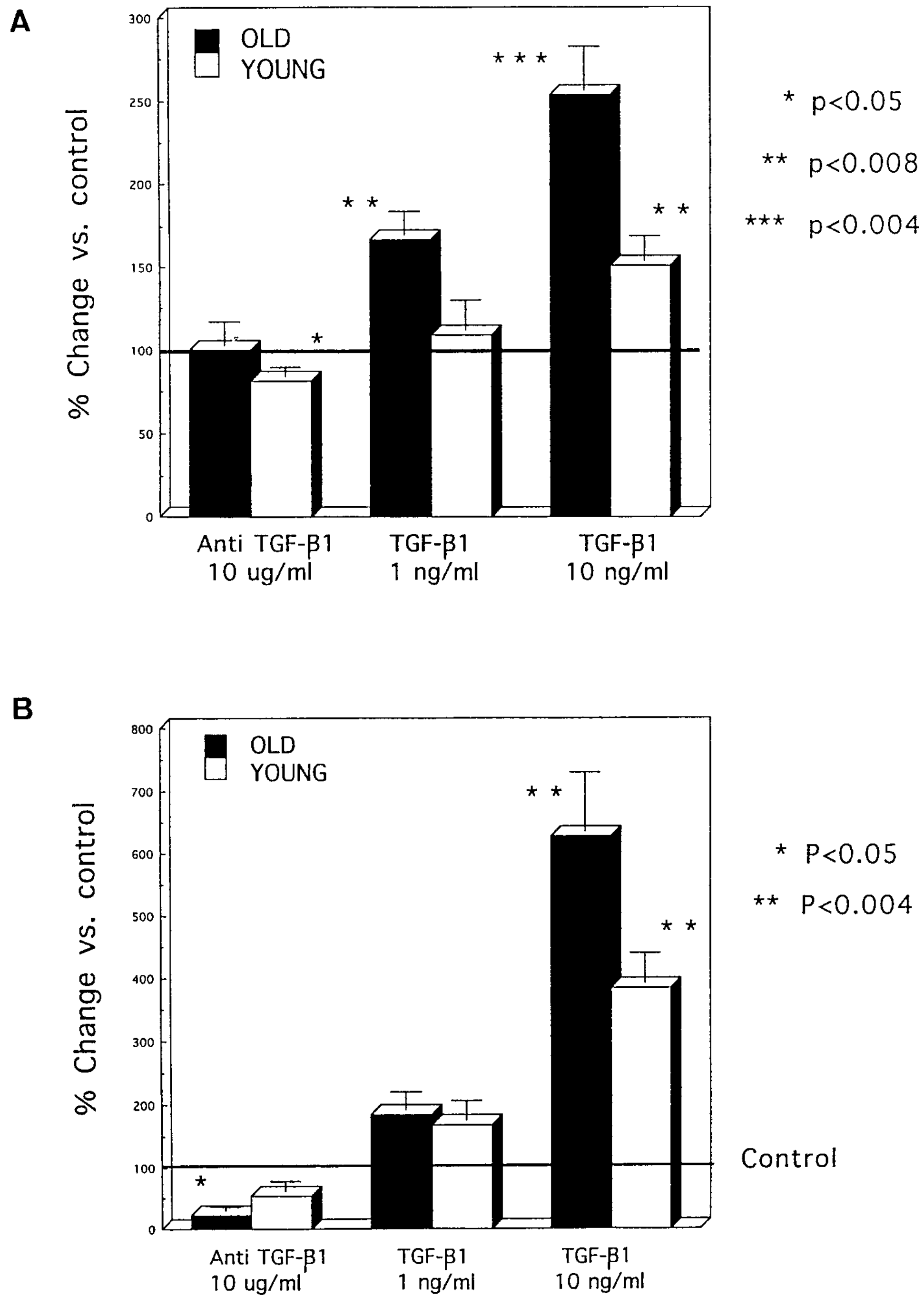


Fig. 5. Endogenous and exogenous TGF-β1 Action on CFU-f colony number (**A**) and size (**B**) of alkaline phosphatase positive CFU-f obtained from young and old mice. CFU-f cultures were supplemented with 20% FCS during the first 48 h and then in reduced serum medium (0.5% FCS) from days 2 to 7. The cultures were treated on days 0, 2, and 5 with 1 ng/ml, 10 ng/ml recombinant TGF-β1 or anti-TGF-β1 antibody. On day 7, the cultures were fixed and stained for alkaline phosphatase activity as described. CFU-f cultured from 24-month-old animals were more sensitive to exogenous TGF-β1 than CFU-f derived from young, 4-month-old mice (A, B). When neutralizing antibody to TGF-β1 was added to CFU-f cultures, the baseline levels of colony formation and proliferative activity were reduced (A, B). The latter effect is most apparent in the number of CFU-f cultures derived from young animals, where the presence of antibody lowered colony number by ~20% (A, statistically significant). In cultures initiated from marrow from old mice, the reduction in size, ~70%, was statistically significant (B). The results represent the mean +/− SE of quadruplicate cultures of one representative experiment of a total of three experiments. All showed identical trends.

loss of bone mass and load-sustaining architecture. These changes are most readily observed in trabecular or spongy bone but are also found in the cortex. The net result of this bone loss in humans when it reaches a sufficient magnitude is osteoporosis, a skeletal disorder characterized by an increased incidence of fractures with sequelae that include pain, loss of mobility, and in the event of hip fracture, death for some individuals [Kanis and Adami, 1994; Johnson and Slemenda, 1995; Baron et al. 1996; also NIH Report "Osteoporosis and Related Bone Diseases," 1996].

The manner in which bone is lost in the osteoporosis (osteopenia) of old age is complex, but at least one important component appears to be a diminution in the number and, perhaps, activity of osteoblasts involved in the synthesis of new bone matrix [Estell et al., 1988; Finkelman et al., 1992; Roholl et al., 1994; Kahn et al., 1995; Bergman et al., 1996]. Whereas this reduction in the number of osteoblasts might occur for a variety of reasons, the findings from this investigation and earlier publications suggest that a major factor is the age-related and significant diminution in the number and osteogenic potential of osteoblast progenitor cells [Owen and Freidenstein, 1988; Kahn et al., 1995; Bruder et al., 1997]. We previously postulated that these regressive ("involutional") changes are the consequence of alterations in the feedback loop between the proliferating and differentiating progenitor cells and a growth factor, TGF-β1, that we believe is essential to these activities. Specifically, we proposed that with advancing age there is a decline in the amount and/or activity of this growth factor.

In a previous study, we showed, using in vitro colony formation assays, that the number and apparent osteogenic activity (i.e., alkaline phosphatase activity) of bone marrow CFU-f is markedly reduced in old male mice [Kahn et al., 1995]. In the present work, we have confirmed and extended this finding by demonstrating that the bone marrow of old animals forms smaller bone ossicles when transplanted under the renal capsule of host mice than similar marrow transplants from young adult animals. Friedenstein [1976] and his colleagues [Freidenstein et al., 1968, 1974] had shown that the ossicles formed from such marrow-to-kidney capsule transplants are derived from donor marrow cells and reflect the number of CFU-f present in the original donor tissue. In addition, we now find significant reductions in the levels of TGF-β in the bone matrix of the femurs and tibias of these same old animals, thereby substantiating, albeit indirectly, that there is less TGF-β synthesized and consequently available to marrow cells in old mice [Nichols et al., 1994; cf. Zeng et al., 1996]. This result is consistent with and supported by yet another finding from the present study, viz, that the osteoblast progenitors (CFU-f) from old mice produce significantly less TGF-β in vitro than similar cells cultured from young animals.

Given this variance in the level of in vitro and, apparently, in vivo TGF-β synthesis by the CFU-f of animals of different age, it is not surprising that there are other growth factor-related differences as well. For example, the number but not affinity of all three types of TGF-β receptor is notably higher in the CFU-f cultured from old animals. This change in receptor level is accompanied by a readily demonstrable increase in the sensitivity of such cells to exogenous recombinant TGF-β1; the addition of growth factor yielding a dose-dependent increase in colony number and size. Perhaps even more informative is the action of neutralizing, anti-TGF-β1 antibody on CFU-f in vitro. The effects of adding this reagent into the culture medium not only further confirms that CFU-f produce the growth factor but also that the cells from young mice are apparently more sensitive to the interruption of endogenous (autocrine) TGF-β1 activity than the corresponding CFU-f from old animals. It seems reasonable to assume that this difference in sensitivity is due to the higher in vitro baseline levels of TGF-β production by the CFU-f from young mice.

Although these results are consistent with the hypothesis that reduced TGF-β1 is an important causative factor in the osteopenia of old age, they are not conclusive. In part, this has to do with the need for an assessment of the action of TGF-β1 in old and young mice in vivo. Some such assessments have now been made and will be presented in detail in Gazit et. al. [unpublished communications]. In brief, the data from this second manuscript show that "replacing" TGF-β1 in old mice by i.p. injection stimulates new bone formation and increases marrow CFU-f number and apparent osteogenic activity. Importantly, such increases in response to growth factor are either not seen or seen only at lower levels in similarly treated young animals.

These additional findings not withstanding, we recognize that growth factors in addition to TGF-β are also involved in the regulation of osteoblast differentiation and note that some of these factors or their binding proteins are also less abundant in advanced age [e.g., Dunstan et al., 1995; Nicolas et al., 1995]. Thus in "replacing" TGF-β? in old animals, it is possible, even likely, that we are setting into motion a cascade of activity involving a number of growth factors, and it is this cascade that leads to osteoblast differentiation and enhanced function.

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