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Recombinant TGF-β1 Stimulates Bone Marrow Osteoprogenitor Cell Activity and Bone Matrix Synthesis in Osteopenic, Old Male Mice

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Abstract We have previously hypothesized that the osteopenic changes seen in the skeletons of old male BALB/c mice are due to reductions in the availability and/or synthesis of bone TGF-β which results in fewer, less osteogenic marrow osteoprogenitor cells (CFU-f; OPCs) and lower levels of bone formation. Among other things, this hypothesis would predict that introducing exogenous TGF-β into old mice (growth factor replacement) should stimulate marrow CFU-f and increase bone formation. In the present study, we have tested this prediction and, indirectly the hypothesis, by injecting human recombinant TGF-β1, i.p., into both young adult (4 month) and old mice (24 month). The effects of the growth factor on the skeleton were then assessed by measurements of trabecular bone volume, bone formation, fracture healing, and the number, proliferative, apoptotic, and alkaline phosphatase activity of marrow CFU-f/OPCs. Our data show that the introduction of 0.5 or 5.0 ug/day of TGF-β1 into old mice for 20 days 1) increases trabecular bone volume, bone formation and the mineral apposition rate, 2) augments fracture healing, 3) increases the number and size of CFU-f colonies, and 4) increases proliferation and diminishes apoptosis of CFU-f in primary bone marrow cultures. Importantly, these stimulatory effects of injected growth factor are apparently age-specific, i.e., they are either not seen in young animals or, if seen, are found at much lower levels. While these observations do not exclude other possible mechanisms for the osteopenia of old mice, they provide further support for the hypothesis that, with age, diminished TGF-β synthesis or availability results in a reduction in the marrow osteoprogenitor pool and bone formation. The findings also demonstrate that the latter changes can be reversed, at least transiently, by introducing exogenous TGF-β1. J. Cell. Biochem. 73:379–389, 1999.

Key words: TGF-β; osteopenia; old mice; marrow osteoprogenitor; bone formation

We previously postulated that the bone loss (osteopenia) associated with old age in mice and other mammals results from an age-related curtailment in the production or availability of TGF-β, a growth factor we believe essential for the differentiation of osteoprogenitor cells (CFU-f or OPCs) in the bone marrow stroma into osteoblasts [Bergman et al., 1995; Bonewald et al., 1994; Centrella et al., 1994; Erlebacher, 1995; Kahn et al., 1995]. As a consequence of this growth factor deficiency, fewer osteoblasts differentiate, matrix synthesis falls below the levels required to replace the bone resorbed during remodeling, and there is a steady loss of bone mass over time [Bergman et al., 1995; Kahn et al., 1995; Manolagas et al., 1995; Tsuji et al., 1990].

To substantiate this hypothesis, it was first necessary to show that there is, in fact, an age-related diminution in both the marrow CFU-f/OPC content and in the bone-matrix associated TGF-β. Documentation of such reductions was obtained in in vitro analyses of cells and tissues from old male mice and has been presented in previous publications [Bergman et al., 1995; Kahn et al., 1995; Gazit et al., 1998]. Since loss of TGF-β appears pivotal to these senescent changes, we wondered whether introducing exogenous growth factor into old mice would reverse these changes by restoring the

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CFU-f/OPC population, stimulating bone formation and increasing bone mass. In the present study, we have done such experiments and find that daily injections of TGF-β1 dose-dependently increase marrow CFU-f number and proliferative activity, promote CFU-f differentiation, decrease CFU-f apoptosis, stimulate bone formation under both physiological and pathophysiological conditions, and increase trabecular bone volume to levels indistinguishable from those observed in 4-month-old mice. Of interest, these latter effects are either not seen or seen at a lower level in TGF-β1 treated young animals.

MATERIALS AND METHODS

Tissue Culture Supplies and Mice

Recombinant human TGF-β1 was obtained from R&D Systems, Inc. (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). Twenty to 24-month-old and 3 to 4-month-old, male Balb/c mice were obtained from Charles River Inc. (Wilmington, MA). In general, eight mice were used in each experimental group and the experiments were repeated on three separate occasions. In animals receiving growth factor, the TGF-β1 was dissolved in PBS containing 0.2% BSA, filter sterilized, and injected, interperitoneally, for 20 consecutive days.

Cell Culture

Modifications of previously described techniques were used to culture stromal CFU-f [Beresford et al., 1994; Cheng et al., 1994; Leboy et al., 1991; Rickard et al., 1994; Tsjui et al., 1990]. Thus, bone marrow was expelled from the tibial and femoral diaphyses using the methods described above. The marrow cells were gently resuspended in medium by passage through a series of 19G, 21G, and 23 G needles, counted and, after appropriate dilution, seeded into 60 mm plastic culture dishes (Nunc, Denmark) at a density of 4×10^6 cells/plate. The cells were then cultured at 37°C in α-MEM medium supplemented with 100 u/ml of Pen-Strep, 50 mg/ml ascorbic acid and 10% FBS. The medium was changed after 48 h of culture and twice a week thereafter, if the cultures were continued for longer than the usual period of time. Typically, however, CFU-f cultures were assayed after 7 days of incubation necessitating only one additional "feeding." Ten mM β-glycerophosphate, 10^{-8} M dexamethasone (Dex) and 50 ug/ml ascorbic acid were added to the medium with the first change of media.

Number, Size, and Alkaline Phosphatase Reactivity of CFU-f

On day 7 of culture, CFU-f in 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). Both total colonies and alkaline phosphatase positive colonies (>50% positive staining cells) were counted. A colony was defined as a group or cluster of cells containing 16 or more members. The scores from each of the six-well plates were averaged for each age group. CFU-f colony size was determined using a computerized morphometric system (Galai: CUE-3 Electro Optical Inspection and Diagnostic Laboratories, Ltd., Migdal Haemek, Israel).

Cell Proliferation

The CFU-f in two, four-well chamber slides (Nunc, Denmark) were incubated for 2 h at 37°C with a bromodeoxyuridine (BrdU) labeling solution, fixed in 70% alcohol, washed with double distilled water, and stained immunohistochemically using the Streptavidin-Biotin system (BrdU Staining Kit, Zymed, Cat 93–3943). BrdU-positive cells were quantified as a percentage of total cells.

Apoptosis

CFU-f in both the early and late stages of apoptosis were detected using ApoAlert Annexin V Apoptosis Kit Clontech, Cat K2025 [Griffith et al., 1995; Martin et al., 1995]. In this assay, cells are incubated in the Annexin V-FITC, 1 ug/ml, and 10 ul of propidium iodide (PI). Early stage apoptotic cells bind Annexin V-FITC and display green staining on the plasma membrane. Late stage apoptotic cells, which have lost membrane integrity, show red (PI) staining throughout the nucleus as well a green staining (from FITC) on the plasma membrane. Late stage cells were also detected by incubating CFU-f in DAPI (4',6'-diamino-2-phenylindole hydrochloride) [Amsterdam et al., 1996, 1997; Keren-Tal et al., 1995]. Cells which contain highly dense and irregular nuclear chro-

matin inclusions are apoptotic when compared to nonapoptotic cells which show homogenous DNA staining throughout the entire nucleus [Amsterdam et al., 1996]. CFU-f pretreated with etoposide (100 ug/ml) to elicit apoptosis in cultured cells served as positive controls. Quantitative determination of the degree of apoptosis was done on seven randomly selected microscopical fields of each well [Keren-Tal et al., 1995]. Data were expressed as means ± SE for each chamber slide.

Histomorphometry

Femora and tibiae were dissected free of soft tissue, fixed in 4% buffered formalin, decalcified, embedded in paraffin, sectioned longitudinally (at 5 um), and stained with H&E and Masson Trichrome. Trabecular bone volume (TBV) was measured in a standardized metaphyseal area (1 mm distal to the lower margin of the epiphyseal cartilage) representing the secondary spongiosa using automatic image morphometrical analysis (Automatic Morphometrical Computerized system, Galai: CUE-3 Electro Optical Inspection and Diagnostic Laboratories, Ltd., Migdal Haemek, Israel). These data were calculated and are presented in Figure 2 as a percentage of total bone marrow space area. For the kinetic analysis of bone formation, the fluorochrome label calcein green (2.5 mg/kg) was administered i.p. at 7 and 2 days prior to sacrifice. Following sacrifice, the tibiae were removed and fixed in 70% ethyl alcohol (EtOH), and embedded in plastic (Immunobed Kit, Polysciences, Warrington, PA). Ten micron unstained sections were evaluated by fluorescent microscopy. Specifically, histomorphometric measurements of the secondary spongiosa of the proximal tibia metaphysis (as noted above, 1 mm distal to the lower margin of the epiphyseal cartilage) were performed with computerized NIH image 1.60 program interfaced via a camera lucida attached to a Nikon light/fluorescent microscope. Total and double labeled areas were measured at a ×25 magnification. Parameters of mineral apposition rate (MAR) [Bain et al., 1993; Rubin et al., 1995]. and bone formation rate were determined at ×100 magnification. Trabecular and endosteal mineral apposition rates (MARt and MARe, respectively) were calculated as previously described [Bain et al., 1993].

Fracture Healing Model

Standard bone fractures were performed on the VIII rib of 12 24-month-old male Balb/c mice [Grills et al., 1995; Nakase et al., 1994]. A skin incision was made in the middle area of the back of an anesthetized mouse (2% Xylazine and 8.5% Ketamine i.p.), the rib separated from surrounding muscles and dissected (fractured) together with periosteum. The position of fractured edges was physiologically fixed by intact adjacent ribs VII and IX. The skin wound was closed with surgical clips. After 3 weeks, the mice were sacrificed, the dorsal parts of the thorax were dissected, fixed in 95% ethyl alcohol (EtOH) followed by clearing in a 20% glycerol/1% KOH solution for 1 week. The cleared tissues were then examined and photographed microscopically. Six of the 12 animals received injections of TGF-β1.

Statistical Analysis

The statistical significance of the results were established using either the Mann-Whitney test or one factor ANOVA [Siegel, 1956]. Data are presented as mean values ± the standard error of the mean.

RESULTS

The Skeletons of Old Male Mice Are Osteopenic

The osteopenic/osteoporotic nature of the skeletons of old male mice is readily apparent in the hemisected femur shown in Figure 1. The bones of the old animals contain conspicuously fewer, thinner, and less well-connected trabeculae (Fig. 1a) than their young mouse counterparts (Fig. 1b). In the latter, the trabeculae have a nearly platelike morphology, reflective not only of a larger amount of bone but also suggestive of greater mechanical strength.

TGF-β1 Increases Trabecular Bone Volume In Vivo in Old But Not Young Mice

TGF-β1 injected, i.p., daily for 20 days markedly increases trabecular bone volume in both the femurs and tibias of old mice. In the representative experiment shown in Figure 2 statistically significant gains in volume are evident at doses of 0.5 and 5.0 ug/day. In the case of the femur, both doses yielded comparable results, viz., an ~3× increase in bone volume (Fig. 2). In the instance of tibias, the 0.5 ug dose improved bone volume ~2× while the higher dose (5.0 ug) increased bone volume ~5× (data not

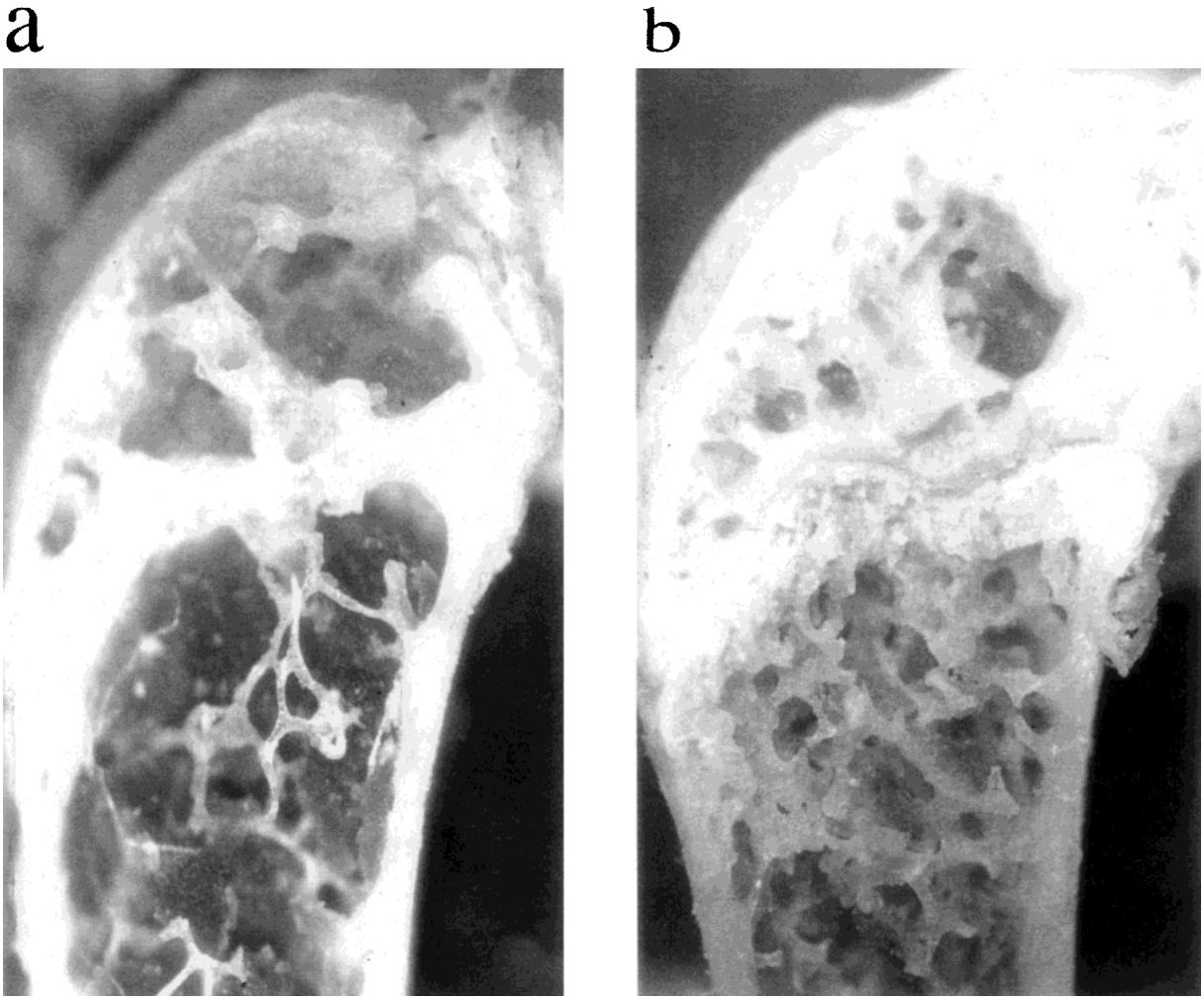

Fig. 1. Longitudinal hemisections of freshly dissected, unfixed femurs obtained from 24-month-old (**A**), and 4-month-old (**B**) male mice. Note the diminution in trabecular number; the thinner and less connected trabecular morphology. The hemisections were made with an ISOMED diamond knife. Original magnification ×3.5.

shown). Importantly, for both the femur and the tibia, the optimal response to TGF-β1 brought bone volumes in treated old mice to levels approximating those seen in young mice under circumstances where no change was observed in comparably treated young animals.

TGF-β1 Treatment Increases Osteoblast Number and Stimulates Bone Formation in Old Mice

Given the above results, it is not surprising to find that injection of TGF-β1 also markedly stimulates the number of osteoblasts and osteoblastic activity in vivo. The increase in osteoblast number (or the number of matrix synthesizing osteoblasts) is suggested by microscopic examination of the bone surface and confirmed by counts of osteoblasts/mm of surface. Thus, while old, untreated animals show no readily identifiable osteoblasts at the light microscope level, mice treated with 0.5 and 5.0 ug/d TGF-β1 for 20 days have 17.5 ± 4.1 osteoblasts/mm of trabecular bone surface. (The data for the two doses were combined because of the similarity of results.) For comparison, young control animals have 30 ± 2.7 osteoblast/mm of surface. The stimulation of bone formation in old mice by TGF-β1 is equally impressive (Fig. 3). At a dose of 0.5 ug/day, the growth factor not only increases the percent of double-labeled surface to the level calculated for young mice (a measurement indicative of the number of osteoblasts involved in matrix synthesis) [Gazit et al., 1995; Manolagas et al., 1995; Rosen et al., 1995], but also markedly augments both the

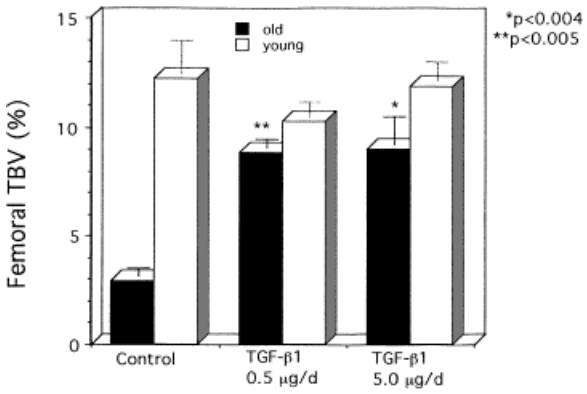

Fig. 2. Histogram representing trabecular bone volume (TBV) in femoral tissue obtained from 24-month-old and 4-month-old male BALB/c mice treated, i.p., with doses of 0.5 and 5.0 ug/day TGF-b1 for 20 days. Note the significant increase ($P < 0.004$ and $P < 0.005$) in trabecular bone volume in old treated animals; volumes which approach those calculated for young mice. On the other hand, the TBV in young treated animals does not show any significant change. These results represent the mean ± SE of 18 animals in three experiments.

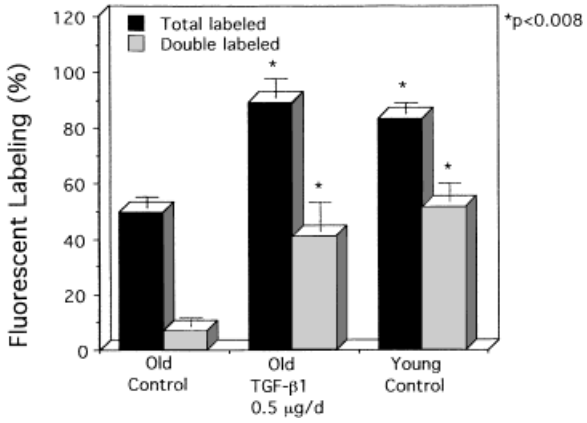

Fig. 3. Histogram representing percent total and double fluorescent-labeled surfaces from the tibial metaphyseal area of 24-month-old untreated BALB/c mice, old animals treated systemically with 0.5 ug/day of TGF-β1, i.p., for 20 days and 4-month-old untreated control mice. Note the significant increase ($P < 0.008$) in the extent of total and double-labeled surfaces (reflective of matrix mineralization) in the TGF-β1 treated mice; values which approach the levels calculated for young mice. The data represent the mean ± SE (n = 8 mice). Data from undecalcified, unstained plastic embedded sections.

mineral apposition rate (MAR) [Bain et al., 1993; Rubin et al., 1995] (Fig. 4.) and bone formation rate (BFR). Thus, the BFR for old, untreated mice is 3.2 ± 0.57 $mm^3/mm^2/day \times 10^{-4}$ while that of old mice treated with 0.5 ug/d growth factor for 20 days is approximately twice as great at 6.3 ± 0.94 $mm^3/mm^2/day \times 10^{-4}$. These values, derived from groups of eight mice,

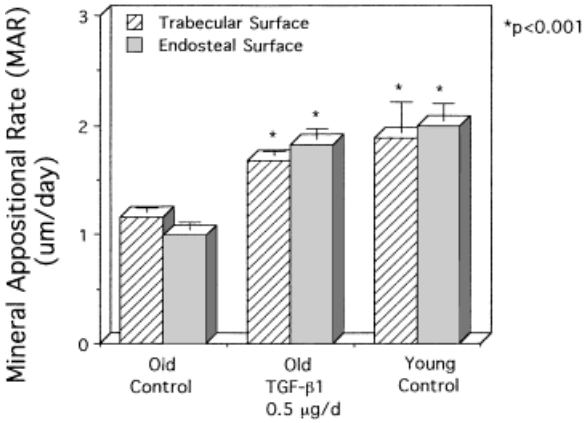

Fig. 4. Histogram showing the mineral apposition rate (MAR) of trabecular and cortical (endosteal) bone in the tibial metaphyseal areas of 24-month-old untreated BALB/c mice, old animals treated with 5 ug/day TGF-b1, i.p., for 20 days and 4-month-old control mice. Note the significant increase ($P < 0.001$) in endosteal and trabecular MAR in TGF-β1 treated mice which approaches the levels calculated for young, untreated animals. The values represent the mean ± SE of eight mice.

are significantly different at $P = <0.001$ level. Collectively, these data clearly demonstrate that the introduction of TGF-β1 in old animals results in more readily identifiable, more cuboidal osteoblasts, and notably higher levels of matrix synthesis and mineralization in both trabecular and endosteal bone.

TGF-β1 Treatment Increases the Extent of Fracture Healing in Old Mice

The stimulation of bone formation in old mice by TGF-β1 is confirmed, within a pathophysiological context, by the improved fracture healing seen in animals treated with the growth factor. As shown in Figure 5, injection of TGF-β1, i.p. at 5.0 ug/day for 20 days stimulates healing of a standard rib fracture with complete bridging of fractured edges by a callus composed substantially of cartilage tissue (Fig. 5b). This is in marked contrast to the minimal healing, with limited callus formation composed mostly of connective tissue (not shown) observed in untreated control, old mice (Fig. 5a). By quantitative measure, the callus formed in treated animals is ~3.5-fold greater in size than that observed in controls. In neither case, however, was new bone formation observed, perhaps because of the relatively limited period of fracture healing at the time the tissue was recovered.

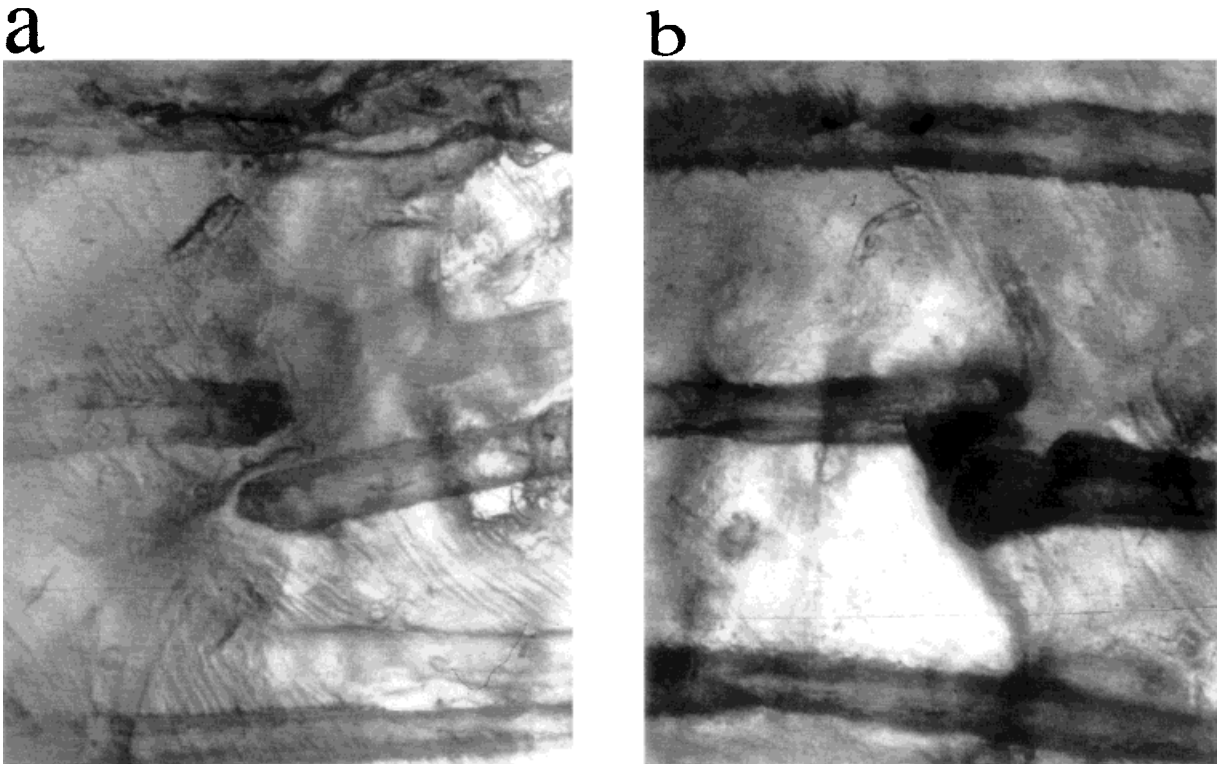

Fig. 5. Photomicrographs of standardized VIII rib fractures as seen in a control 24-month-old, male BALB/c mouse (**a**) and an old male mouse treated with 5.0 ug/day of TGF-β1, i.p., for 20 days (**b**). Note the active healing process with callus formation bridging the edges of the fractured rib in the TGF-β1 treated, old animal. These observations are representative of the results from six untreated and six treated mice.

TGF-β1 Treatment In Vivo Increases Colony Number and Size and Cell Proliferative Activity of Marrow CFU-f But Inhibits Apoptosis

If there is a correlation between growth factor-stimulated bone formation and marrow CFU-f, it should be apparent in CFU-f isolated from the marrow of TGF-β1-treated old mice. That this is indeed the case is shown in Figure 6a,b. Injection of either 0.5 or 5.0 ug TGF-β1/day for 20 days significantly increases the number and size, i.e., percentage of culture plate covered, of ALP-positive colonies (Fig. 6a,b). At the lower, 0.5 ug dose, the increase in colony number was ~2.5× and size ~1.7× ($P < 0.001$ vs. that of untreated controls).

Given these increases at the level of colony measurements, it is not surprising that the proliferation of CFU-f is also stimulated by TGF-β1 treatment. As shown in Figure 7a, the percentage of BrdU positive (S phase) cells is ~3× higher ($P < 0.05$) in CFU-f isolated from mice receiving 0.5 ug/day of growth factor than in CFU-f cultured from control animals. Conversely, the fraction of cells undergoing apoptosis is markedly reduced in CFU-f cultured from treated animals. This is shown in Figure 7b,c. These latter observations reflect measurements of apoptotic activity at both early and late stages of cell death.

DISCUSSION

TGF-β is a highly potent growth factor that is produced in multiple tissues and is known to affect the function of numerous different cell types [Bonewald et al., 1995; Carlino et al., 1992; Centrella et al., 1994; Erlebacher et al., 1995; Finkelman et al., 1992; Hestdal et al., 1993; Jennings et al., 1988; Manolagas and Jilka, 1995; Shull et al., 1992]. However, its action on target tissues is complex leading, not infrequently, to apparently contradictory findings. For example, the collective data on TGF-β and skeletal tissue generally suggest that the growth factor stimulates cell proliferation and extracellular matrix production by osteoblastic cells [Bonewald et al., 1994; Centrella et al., 1994; Westerlind and Turner, 1991] but inhibits the formation and bone-resorbing activity of

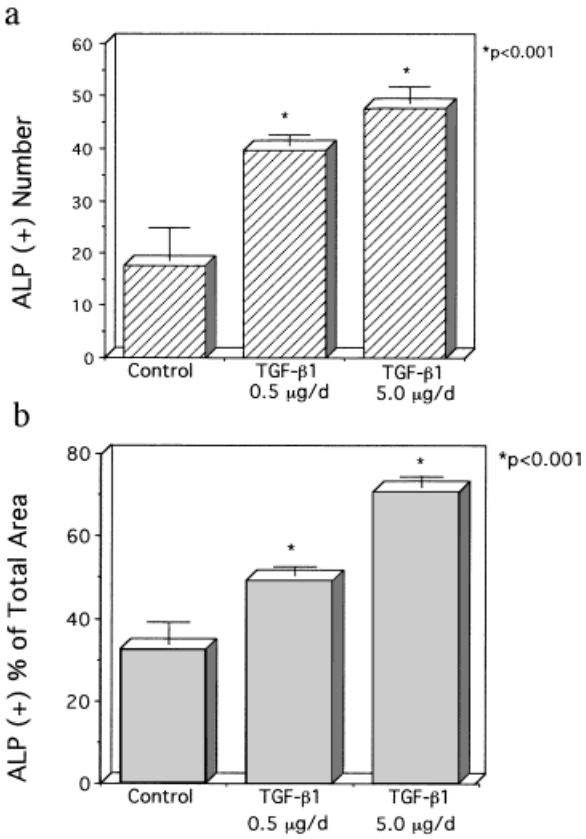

Fig. 6. **a**: Histogram showing the number of alkaline phosphatase (ALP) positive CFU-f colonies in 10-day-old primary cultures established from the bone marrow of 24-month-old mice treated, in vivo, with either 0.5 or 5.0 ug/day, i.p., for 20 days prior to tissue collection. Note the significant ~2.5× increase in ALP(+) colony number ($P < 0.001$ vs. nontreated controls). **b**: Histogram representing the size of alkaline phosphatase (ALP) positive CFU-f colonies in 10-day-old primary cultures derived from the bone marrow of 24-month-old mice treated with either 0.5 and 5.0 ug/day of TGF-β1 in dose, i.p. for 20 days. Note the significant ~1.7× increase in ALP(+) colony size ($P < 0.001$ vs. cultures from untreated control animals). The values shown represent the mean ± SE in triplicate cultures from three experiments.

osteoclasts [Beaudreuil et. al., 1995; Manolagas and Jilka, 1995]. However, for these "anabolic" effects on osteoblastic cells to occur, there has to be a concordance of number of variables including the stage of differentiation of the target cells and the manner of exposure (e.g., continuous vs. intermittent) and concentration of growth factor. Thus, relative to the issue of the stage of differentiation, TGF-β appears to stimulate the proliferation of relatively immature osteoprogenitor cells but to curtail or inhibit the latter stages of differentiation [e.g., Bonewald et al., 1990; Critchlow et al., 1995; Gazit et al., 1993; Harris et al., 1994; Mundy and Bonewald, 1992]. In at least one in vitro model of osteoblast differentiation, the problem of late stage inhibition by TGF-β appears to have been solved by the downregulation the TGF-β receptor in more mature cells [Gazit et al., 1993].

The CFU-f resident in bone marrow include a stem cell population with intrinsic osteogenic potential. This potential was demonstrated most comprehensively in the studies of Freidenstein and his colleagues who found that marrow CFU-f, explanted to ectopic sites in suitable host animals develop bony, remodeling ossicles in the absence of an apparent bone inductive stimulus [Freidenstein et al., 1968, 1974; Owen, 1988; Owen and Friedenstein, 1988]. From this result, they formulated the notion that the marrow CFU-f included "determined osteogenic precursor cell(s)" (DOPCs), i.e., progenitor cells intrinsically capable of differentiating into chondrocytes and osteoblasts [Freidenstein et al., 1968]. Since such committed osteogenic precursor cells appear to represent the kind of target that is positively responsive to TGF-β action (see above), it follows that this growth factor might play a particularly important role in marrow CFU-f development and differentiation.

Although TGF-β1 is one of the most abundant growth factors in the bone extracellular matrix, the absolute amount may vary as a function of age or physiological circumstance. For example, Nicolas et al. [1994] found that the level of TGF-β in matrix is significantly reduced in the bones of older, post-postmenopausal women when compared to their younger counterparts. The same group also reported that TGF-β abundance in bone matrix declines in rats following ovariectomy but is restored after estrogen treatment [Finkelman et al., 1992]. Finally, other investigators have reported marked reductions in TGF-β mRNA levels in the osteoblasts of old rats [Manolagas and Jilka, 1995].

There have been previous reports of age-related reductions in the developmental potential of the marrow stromal CFU-f population and human calvarial cells, and of a more limited ability of these cells to undergo osteogenesis [Bergman et al., 1995; Inoue et al., 1997; Pollak et al., 1997; Quarto et al., 1995; Tsuji et al., 1990]. Of these latter studies, perhaps the most representative are the findings of Tsuji et al. [1990], which showed a marked age-related reduction in the capacity of rat marrow stromal

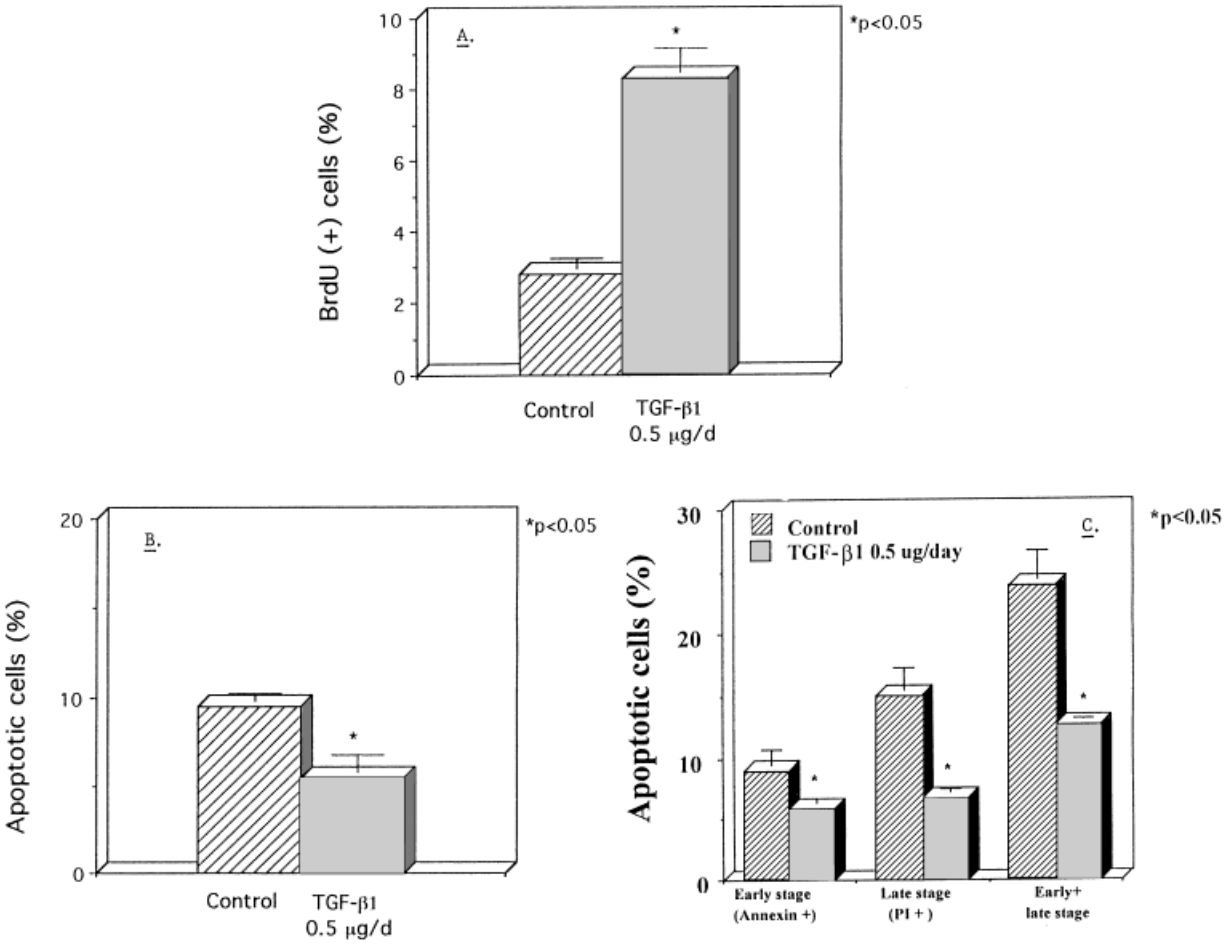

Fig. 7. Treatment of 24-month-old mice with 0.5 ug/day (i.p.) rhTGF-β1 for 20 days in vivo increases proliferation and diminishes apoptosis in CFU-f as assessed in vitro. **a**: Histogram showing the percent of BrdU positive cells in CFU-f primary cultures incubated for 10 days. Note the significant increase in the percentage of BrdU positive cells (~3.0×, $P < 0.05$ vs. cultures from untreated control animals) indicative of a stimulation in cell proliferation. The values shown represent the mean ± SE of duplicate cultures from two experiments. **b**: Histogram depicting the percentage of apoptotic cells in 10 day cultures as assessed by DAPI staining. Note the significant drop in the percent of apoptotic cells (~1.7×, $P < 0.05$ vs. untreated controls). These results represent the mean ± SE of duplicate cultures of two experiments. **c**: Histogram showing the percent of apoptotic cells in 10 day primary cultures of marrow CFU-f. Early apoptotic stages are detected with Annexin V, late stage cells by staining with propidium iodide. Note the significant reduction in treated mice ($P < 0.05$) in the percentage of apoptotic cells at both the early and late stages of programmed cell death.

cells to form mineralized nodules in vitro and Quarto et al. [1995] who found that the in vitro limitations in marrow cell colony forming capacity in old rats is paralleled by a diminished ability to form bone when implanted in vivo. It was from such observations, together with reports of a reduction in bone TGF-β level with age and/or menopausal status (above) and our initial findings of lower CFU-f numbers in old male mice [Kahn et al., 1995], that we formulated our hypothesis linking TGF-β level and/or activity age-related changes in marrow CFU-f osteoprogenitor (OPC) cells. Thus, according to this view, the loss of trabecular bone observed in old mice (and, presumably, in humans as well) is due, at least in part, to what is essentially a TGF-β deficiency. In the circumstance of insufficient TGF-β, osteoblast differentiation would be curtailed (fewer osteoblasts would produce less bone matrix), leading to a decline in bone forming activity.

Recently, we confirmed a lower CFU-f/OPC level in the marrow of old male mice by showing that the bone marrow of such animals produces substantially smaller bone ossicles when transplanted under the renal capsule than the marrow of young mice [Gazit et al., 1998]. We also demonstrated that the bone matrix of femora

and tibiae of old mice contains lower levels of TGF-β, and that the CFU-f from such animals produce significantly smaller amounts of TGF-β, as assessed in vitro. Additionally, we observed that 1) there is an upregulation of all three types of TGF-β receptor in the CFU-f cultured from old mice, 2) that these cells are more responsive than cells from young donors to exogenous TGF-β, and 3) that this growth factor appears to be involved in the autocrine regulation of CFU-f colony formation and cell proliferation as expressed in vitro.

In the present investigation, we extended our analysis of TGF-β1 to the whole animal and attempted to test a major prediction that seems to follow from the hypothesis, viz., that if the reduced CFU-f/osteoblast bone forming activity seen in old male mice is principally the result of diminished TGF-β availability, then providing such animals with a new source of growth factor should reverse these changes. Indeed, the latter is what we seem to have found. TGF-β1 injected into old male mice increases the amount of trabecular bone, stimulates bone matrix synthesis and mineralization, increases the number and proliferative activity of marrow CFU-f, and decreases CFU-f apoptosis. The latter finding is of interest since it was recently shown that the growth factor *promotes* apoptosis in mouse osteoclasts [Hughes et al., 1996]. Thus, as we suggested previously, the same age-related circumstance of reduced TGF-β which might lead to diminished osteoblast activity (bone formation) might also lead to increased osteoclast activity (bone resorption); the two reciprocal situations leading to even further decreases in bone mass [Rickard et al., 1994]. Importantly, this effect of reintroducing TGF-β1 seems age specific; as described above, young adult male mice, injected with the growth factor, either do not respond or do so at a much reduced level. The bones of these still growing animals "behave" as if they were already functioning at or near optimal levels from endogenously available TGF-β.

The finding that overproduction of TGF-β2 by osteoblasts in transgenic mice results in a hyperparathyroidism or osteoporosis-type phenotype [Erlebacher et al., 1996] do not necessarily negate our results. Indeed, in a more recent and very comprehensive study also using TGF-β2 transgenics, this same group showed convincingly that this growth factor directly increases the steady-state differention of osteoblasts (into osteocytes) and, at least indirectly, promotes bone matrix synthesis [Ehlebacher et al., 1998]. In any case, as noted previously, the effects of TGF-β on osteoblasts is variable depending upon the dose and duration of exposure to growth factor and the maturational stage of the target cell. Moreover, as we have seen in this and previous studies [Gazit et al., 1998], the age of animals can play a major role in determining the response to TGF-β, at least to growth factor levels which exceed the amounts normally available physiologically. Finally, we have to consider the possibility that two different isotypes of TGF-β, β1, and β2, were used in the present and the Erlebacher et al. [1996, 1998] studies and that the actions of these two, very similar proteins may not be identical in vivo.

We find these results encouraging and, to date, consistent with the original hypothesis. However, we are aware that there are other important issues that need to be resolved. Thus, it is important to establish the relationship, if any, between TGF-β and other growth factors important in bone metabolism that may be also diminished in level or activity with age. For example, the study of Nicolas et al. [1994], referred to above, reports a marked age-related diminution in bone matrix of IGF-I; a reduction that was subsequently correlated with a loss of matrix-associated IGF binding protein, IGFBP-5 [Nicholas et al., 1995]. Thus, it might be asked, is it the loss of the two growth factors, in tandem, that is ultimately responsible for the osteopenia of old age or is it really a matter of loss of sequence, one growth factor being needed to trigger the action of the second? On a simpler level, we need to determine whether the effects of TGF-β on the skeleton of old animals persist beyond the treatment period and, if so, for how long. We also need to assess whether old female animals are responsive to exogenous growth factor and whether TGF-β1 is more potent (or specific) than other anabolic agents in reversing skeletal change. Finally, we need to explore the mechanism by which TGF-β1 levels are reduced with age and to determine whether the skeletal effect of TGF-β, in vivo, is solely by the directly action on target cell (e.g., the CFU-f/marrow stromal cell) or works, at least in part, by some indirect route.

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