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Independent Changes in Type I and Type II Receptors for Transforming Growth Factor β Induced by Bone Morphogenetic Protein 2 Parallel Expression of the Osteoblast Phenotype

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Transforming growth factor β (TGF-β), a potent regulator of bone formation, has bifunctional effects on osteoblast replication and biochemical activity that appear differentiation dependent. We now show that cell surface binding sites for TGF-β vary markedly among fibroblasts, bone-derived cells, and highly differentiated osteosarcoma cultures from fetal rats. Expression of betaglycan and type II receptors decline relative to type I receptor expression in parallel with an increase in osteoblast-like activity, predicting that the ratio among various TGF-β binding sites could influence how its signals are perceived. Bone morphogenetic protein 2 (BMP-2), which induces osteoblast function, does not alter TGF-β binding or biochemical activity in fibroblasts and has only small effects in less differentiated bone cells. In contrast, BMP-2 rapidly reduces TGF-β binding to betaglycan and type II receptors in osteoblast-enriched primary cell cultures and increases its relative binding to type I receptors in these cells and in ROS 17/2.8 cultures. Pretreatment with BMP-2 diminishes TGF-β-induced DNA synthesis in osteoblast-enriched cultures but synergistically enhances its stimulatory effects on either collagen synthesis or alkaline phosphatase activity, depending on the present state of bone cell differentiation. Therefore, BMP-2 shifts the TGF-β binding profile on bone cells in ways that are consistent with progressive expression of osteoblast phenotype, and these changes distinguish the biochemical effects mediated by each receptor. Our observations indicate specific stepwise actions by TGF-β family members during osteoblast differentiation, developing in part from changes imprinted by BMP-2 on TGF-β receptor stoichiometry.

Transforming growth factor type β (TGF-β) is a ubiquitous growth regulator (14). Several closely related TGF-β isoforms are found in mammals, and, except in rare instances, all bind to the same or overlapping sets of cell surface binding sites (38, 39). Recent cloning and reexpression studies in continuous epithelial cell cultures with chemical-induced mutations indicate that 53-kDa (type I) and 73-kDa (type II) receptors transduce biological signals, while >200-kDa cell surface proteoglycans (type III sites, termed betaglycans) may accumulate and present TGF-βs to signaling receptors (30, 34, 39, 54, 55). Simultaneous association of ligand with select subsets of binding sites appears to be necessary for some biological effects (17, 23, 33, 42, 54), although the relative expression of these sites varies among many cells and species (39). However, some studies indicate that certain effects by TGF-βs result from differential binding to one or another receptor (3, 11, 20, 22, 25).

In skeletal tissue, TGF-βs are highly concentrated and produce complex stimulatory and inhibitory effects on bone cell function. These differences appear to depend on TGF-β concentration, duration of exposure, and present state of cell differentiation (reviewed in reference 7). For example, cells found in fetal and newborn rodent calvarial bone express different degrees of osteoblast-like activity and display a graded response to TGF-β treatment (8, 29, 40). TGF-βs have weak mitogenic effects in less differentiated periosteal cells, potently enhance replication in fetal osteoblast-enriched cultures (8, 29), and inhibit replication by more mature, highly differentiated bone cells (5, 44). Initial receptor characterization studies revealed proportionally less TGF-β binding to type II receptors and betaglycans, by comparison with type I receptors, on more differentiated bone cells and only a single class of binding sites on highly differentiated clonal rat osteoblasts (5, 9, 24). These findings predict that a redistribution in TGF-β binding among various sites could account for the diversity of its biological effects previously detected in bone cell cultures at different stages of phenotype progression. In this regard, treatment with hormones that either increase relative ligand binding to betaglycan, reduce binding to type I or type II receptors, or produce combinations of these changes decrease TGF-β effectiveness (9, 10).

Other members of the TGF-β supergene family with important effects on bone cell function are the bone morphogenetic proteins (BMPs). First defined functionally, BMPs initiate cartilage formation and osteogenesis when implanted with demineralized bone matrix at ectopic sites in rats in vivo. At least seven BMPs with TGF-β gene-related sequences have been reported, and some appear to mimic or to oppose the effects of TGF-βs in various bone and bone cell culture models. Although BMPs alone may initiate ectopic bone formation in vivo, these observations do not eliminate requirements for or interactions with other growth factors supplied by the organism (reviewed in references 7 and 50). While they are closely re-

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lated, the BMPs do not efficiently compete with TGF-βs for primary TGF-β binding sites. Nonetheless, initial studies suggest that BMP receptors may be similar in sequence or function to TGF-β type I and type II receptors (4, 18, 43, 52).

To understand how variations in TGF-β binding sites correspond to osteoblast differentiation, we compared their biological effects and cell surface binding profiles in fetal rat fibroblasts and bone-derived cells expressing progressive degrees of osteoblast-like activity. We then attempted to integrate this relationship with situations in which osteoblast-like function was increased by BMP-2 and to utilize this information to specify the possible role of individual TGF-β receptors on bone cells.

MATERIALS AND METHODS

Cell cultures. Primary cultures were prepared from parietal bones of 22-day-old rat fetuses [stock designation Crl:CD(SD)BR, derived from Sprague-Dawley rats, Charles River Breeding Laboratories, Raleigh, N.C.]. Bone explants were dissected free of sutures and digested for five 20-min intervals with collagenase. Cells in population 1, released in the first collagenase digestion period, exhibit fewer osteoblast-like characteristics than those in population 3-5, released during the last three digestions (8, 40). Cells from population 1 and a pool of cells from population 3-5 were plated at 6×10^3 to 9×10^3 cells per cm^2 in Dulbecco's modified Eagle's medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.2), 100 μg of ascorbic acid per ml, penicillin and streptomycin, and 10% fetal bovine serum. At confluence (about 6.0×10^4 cells per cm^2), they were refed identical medium lacking fetal bovine serum. Twenty hours later, they were refed serum-free medium containing various agents for the times indicated for each experiment. Fetal rat dermal fibroblasts were prepared from skin taken directly above the parietal bones. NRK-49F fetal rat fibroblasts (stock designation CRL 1570; obtained from the American Type Culture Collection) and clonal osteosarcoma-derived osteoblast-like ROS 17/2.8 cultures (obtained from Masaki Noda and Gideon Rodan, Merck Sharp and Dohme Research Laboratories, West Point, Pa.) were cultured and treated by identical procedures.

Reagents. Cell culture reagents were obtained from GIBCO (Grand Island, N.Y.). Recombinant human BMP-2 was prepared and purified to homogeneity in our laboratories at Genetics Institute, Inc. Native platelet-derived TGF-β was obtained from several commercial sources (Upstate Biotechnology, Inc., Lake Placid, N.Y.; Collaborative Research, Inc., Bedford, Mass.; or R&D Systems, Inc., Minneapolis, Minn.), and all preparations produced similar results.

Cell replication. Vehicle or test agent was added in serum-free medium, and cultures were incubated for additional intervals, designated for each figure. DNA synthesis rate was measured by labeling with 5 μCi of [*methyl*-^{3}H]thymidine (80 Ci/mmol) per ml during the last 2 h of culture, lysing the cells in 0.1 M sodium dodecyl sulfate–0.1 N NaOH, collecting the insoluble material formed by precipitation with 10% trichloroacetic acid, and scintillation counting. Data are shown as total [^{3}H]thymidine incorporated per culture (8).

Collagen synthesis. Cultures were pulsed with 12.5 μCi of [2,3-^{3}H]proline (2.5 Ci/mmol; DuPont NEN, Boston, Mass.) per ml for the last 2 h of culture. Cell layers were lysed by freeze-thawing and extracted in 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). The samples were precipitated with 10% trichloroacetic acid and chilled, and the acid-precipitable material was collected by centrifugation. Precipitates were acetone extracted, dried, resolubilized in 0.5 N acetic acid, and neutralized with NaOH. [^{3}H]proline incorporation into collagen (collagenase-digestible protein) was measured by using bacterial collagenase free of nonspecific protease activity and is shown as the total amount of [^{3}H]proline incorporated per culture (6, 8, 47).

Alkaline phosphatase activity. Enzyme activity was assessed in extracts prepared from 2-cm^2 cultures (<1% of total activity is released to the medium; unpublished result) by lysis in 0.5% Triton X-100. Hydrolysis of *p*-nitrophenylphosphate was measured at 410 nm following 30 min of incubation at 37°C (35). Data are expressed as picomoles of *p*-nitrophenol released per minute per microgram of cell protein, determined by the method of Bradford (2).

Binding studies. TGF-β1 radioiodinated with chloramine T to a specific activity of 4,500 Ci/mmol was obtained from Biomedical Technologies, Inc., Stoughton, Mass. Binding was examined by incubation with serum-free medium containing 4 mg of bovine serum albumin per ml and 50 to 150 pM ^{125}I-TGF-β1 for 3 h at 4°C. To visualize TGF-β binding complexes, cultures were rinsed with chilled binding medium, cross-linked with 0.2 mM disuccinimidyl suberate, extracted, and fractionated by electrophoresis on a 5 to 10% gradient polyacrylamide gels, and bound ^{125}I-TGF-β1 was visualized by autoradiography (5, 9, 10). Densitometry was assessed with a Molecular Dynamics densitometer equipped with ImageQuant software.

Northern (RNA) analysis. To assess type I TGF-β receptor, we used a 1.0-kbp *Eco*RI fragment of a cDNA clone (*Alk-5*) containing C-terminal sequences of the kinase domain through 3′ untranslated sequences, provided by K. Miyazono and P. tenDijke (23). To assess a second type I receptor species, we used a 2.4-kbp *Eco*RI restriction fragment obtained from plasmid containing full-length information (*Tsk 7L*), provided by R. Ebner and R. Derynck (17). Type II TGF-β receptor was analyzed with a 4.7-kbp *Eco*RI fragment released from a plasmid containing full-length receptor coding sequence (*H2-3FF*), provided by R. A. Weinberg (31). Betaglycan transcripts were assessed with an *Eco*RI restriction fragment of plasmid BG7 (a subclone of λ*bg7*), containing 3.9 kbp of full-length coding information, provided by F. Lopez-Casillas and J. Massague (32). Total cellular RNA was extracted with acid guanidine-monothiocyanate, precipitated with isopropanol, and heat denatured in 2.2 M formaldehyde–12.5 M formamide at 60°C. Equal amounts of RNA were fractionated on a 1.5% agarose–2.2 M formaldehyde gel. Molecular size was determined by comparison with RNA standards of 0.24 to 9.5 kb (BRL, Gaithersburg, Md.). Gels were blotted onto BioTrace RP charged nylon (Gelman Sciences, Inc., Ann Arbor, Mich.), and membranes were hybridized with cDNA restriction fragments radiolabeled with [α-^{32}P]dCTP and [α-^{32}P]TTP by the random hexanucleotide-primed second-strand synthesis method. Bound material was visualized by Cronex-enhanced autoradiography (DuPont, Wilmington, Del.), and rRNA bands were visualized by ethidium bromide staining of a separate gel containing an aliquot from each sample as previously reported (13, 21, 41).

Statistical analysis. Data were analyzed in multiple samples after multiple determinations and are expressed as means ± standard errors (SE), as indicated in the figure legends. When more than one concentration of variable or more than one group were compared, statistical differences were assessed by analysis of variance, with limits set by the Bonferroni or Dunnet procedure, as each situation required, and performed with a commercial statistical software package. Differences were considered significant with P values of ≤ 0.05.

RESULTS

TGF-β biological activity. TGF-βs have diverse effects on proliferation, matrix synthesis, and phenotype expression in mesenchymal tissue-derived cells, such as those from bone (5–10, 14, 24, 29, 44, 45). Figure 1 contains representative results with fetal rat fibroblasts and bone cells that express various levels of osteoblast-like activity. After a short serum deprivation, TGF-β1 does not significantly alter fibroblast replication in confluent cultures, analogous to early evidence reported with these cultures (36, 37, 48). However, TGF-β1 enhances DNA synthesis in primary cultures obtained from fetal rat bone. A weaker mitogenic effect occurs in less differentiated (population 1) bone cells than in osteoblast-enriched (population 3-5) cultures, while TGF-β1 inhibits DNA synthesis in highly differentiated ROS 17/2.8 osteoblasts. In contrast, TGF-β1 increases new collagen synthesis in each of these four cell models. The highest overall rate of collagen synthesis was seen in TGF-β-treated ROS 17/2.8 cultures, although the relative effect of TGF-β was somewhat less, perhaps because of the high basal levels that already occur in unstimulated cells. Lastly, TGF-β1 reduces alkaline phosphatase in primary population 1 and population 3-5 bone cell cultures but increases enzyme activity in ROS 17/2.8 cells. These changes in proliferation and differentiation appear to follow the sequential patterns observed within early stages of the osteoblast-like development (26, 51).

TGF-β binding site profiles. Differences in TGF-β action in these culture models could result from changes in the expression or the capacity of select TGF-β binding proteins or receptors. A direct comparison of ^{125}I-TGF-β1 binding by these cells revealed subtle variations and some conspicuous differences among the three well-characterized type III (betaglycan), type II, and type I binding sites (38, 39). The TGF-β1 binding profile on fetal rat fibroblasts was similar to the pattern seen on population 1 cells, although there was less relative binding to betaglycan in population 1, in which the complexes migrated with a slightly greater molecular mass. Using the type I receptor as a basis of comparison, we found an even greater apparent decrease in relative binding to betaglycan on population 3-5 cells, while type I receptors were the most evident species in highly differentiated ROS 17/2.8 cultures (Fig. 2). Longer gel exposures sometimes increased the detection of

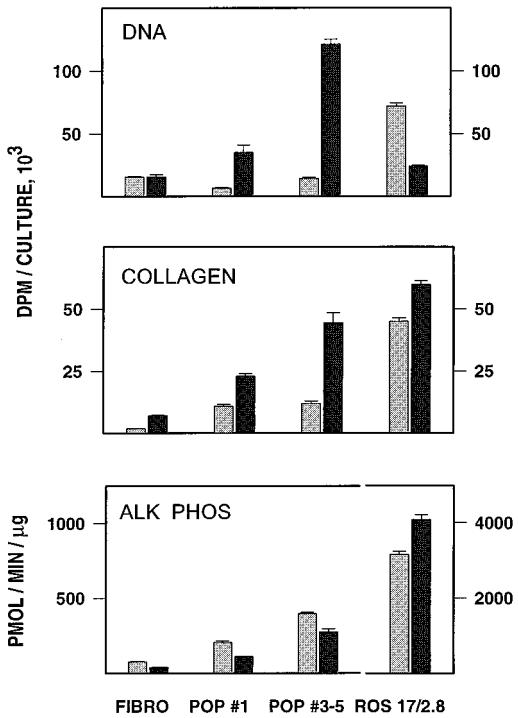

FIG. 1. TGF-β effects on DNA and collagen synthesis and on alkaline phosphatase activity in fetal rat cell cultures. Serum-deprived cultures were treated for 23 h with a maximally effective concentration of TGF-β1. DNA synthesis was determined by labeling with [^{3}H]thymidine during the last 2 h of treatment and by acid precipitation. Collagen synthesis was determined by labeling with [^{3}H]proline during the last 2 h of treatment and by differential sensitivity to purified collagenase. Alkaline phosphatase was measured by *p*-nitrophenylphosphate hydrolysis. Data are means ± SE for four to six replicate samples per condition. Similar effects were found in a minimum of three separate studies. TGF-β1 at concentrations of 0.004 to 0.12 nM has biphasic effects on DNA synthesis (see Fig. 7) and linear effects on collagen synthesis (see Fig. 8) and alkaline phosphatase (5, 6, 8–10). Therefore, effects shown in the top panel were determined with 0.04 nM TGF-β1 (maximal for effects on DNA synthesis), and those in the middle and bottom panels were determined with 0.12 nM (maximal for effects on collagen synthesis and alkaline phosphatase). TGF-β1 induced significant changes in DNA synthesis and alkaline phosphatase activity in population 1 (POP #1), population 3-5 (POP #3-5), and ROS 17/2.8 cultures and significantly enhanced collagen synthesis in all cultures, including fetal rat fibroblasts (FIBRO). Analogous changes occurred in continuously cultured NRK-49F fetal rat fibroblasts or in primary fetal rat dermal fibroblast cultures (not shown). ▭ , Control culture; ⊠ , TGF-β-treated culture.

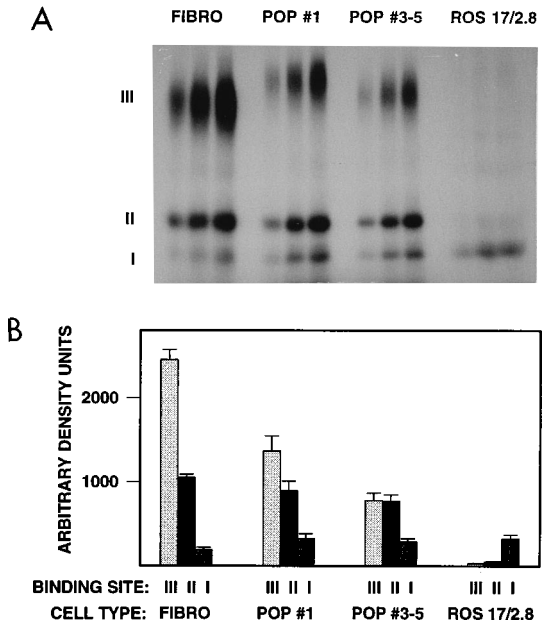

FIG. 2. TGF-β1 binding profiles in fetal rat cell cultures. Extracts from serum-deprived cultures bound and cross-linked to ^{125}I-TGF-β1 were fractionated on 5 to 10% polyacrylamide gels, visualized by fluor-enhanced autoradiography (A), and analyzed by densitometry (B). To obtain patterns in the linear range of autoradiography, 3 volumes of extract (one-third, two-thirds, or the entire amount) from a 9.6-cm^2 culture from each cell model was evaluated. First three lanes, NRK-49F fetal rat fibroblasts; second three lanes, population 1; third three lanes, population 3-5; last three lanes, ROS 17/2.8. Roman numerals on the left indicate the TGF-β receptor class. Complexes were designated by analogy to other tissue systems, in which the type III complex migrates at >200 kDa, the type II complex migrates at 85 kDa, and the type I complex migrates at 65 kDa (38), relative to a mixture of protein standards.

faint bands in the range of 75 kDa and 130 to 175 kDa in ROS 17/2.8 extracts, but proteins comigrating with conventional ^{125}I-TGF-β1 labeled complexes of 85 kDa (type II receptor) or >200 kDa (betaglycan) were never easily observed.

TGF-β binding site mRNAs. The most obvious differences among the bone cell cultures related to the loss of betaglycan and type II receptors and to the retention of type I receptors as expression of osteoblast-like function increased. Using equivalent amounts of total RNA from each culture model, we observed by Northern analysis a predominant 6.1-kb RNA band and a less intense 3.7-kb RNA band that hybridized with betaglycan core protein cDNA (*bg7* [32]). More of each RNA species occurred in fetal rat fibroblasts and population 1 cultures than in population 3-5, and negligible levels were found in ROS 17/2.8, analogous to the decreasing amounts of cell surface betaglycan seen on these cells. Parallel RNA blots probed with type II TGF-β receptor cDNA (*H2-3FF* [31]) revealed a principal RNA species at 4.8 kb, migrating slightly below 28S rRNA (at 5.1 kb), and a trend in relative steady-state transcript levels similar to that of betaglycan mRNA. Probing with type I TGF-β receptor cDNA (*Alk-5* [23]) demonstrated bands of 6.0, 3.5, and 1.6 kb, with the intermediate band predominating. In these four cultures, the relative expression pattern of the 3.5-kb type I receptor transcript was inverse to that of betaglycan and type II receptors (Fig. 3). Also, hybridization with cDNA encoding an alternate type I receptor protein (*Tsk 7L* [17]) that binds both activin and TGF-β (23) revealed a major transcript of 3.4 kb (determined by migration in ROS 17/2.8 extracts, which contained the least abundant transcript levels). Among these cultures, the pattern of transcripts hybridizing with *Tsk 7L* was dissimilar to that with the other cDNA probes. Although type II and both type I TGF-β receptor species contain kinase domains with similar coding sequences that could overlap in part during transcript analysis, the very distinct hybridization patterns that we observed in parallel blots assessed under high-stringency conditions are inconsistent with cross-hybridization (Fig. 3).

BMP biological effects. Since BMPs enhance phenotype development in several cell culture models associated with chondrogenesis and osteogenesis (12, 28, 50, 53), we suspected that the progressive changes in osteoblast-related biological activity that we noted could be further driven by these agents. BMP-2 treatment had no mitogenic effects in continuously cultured NRK-49F fibroblasts (Fig. 4) and only weak activity in fetal rat dermal fibroblasts (not shown) but enhanced biochemical activity in primary cell cultures from fetal rat bone. Unlike TGF-β1, BMP-2 potently enhanced DNA synthesis, collagen synthesis, and alkaline phosphatase activity in population 1 and in population 3-5. Relatively greater increases in DNA synthesis

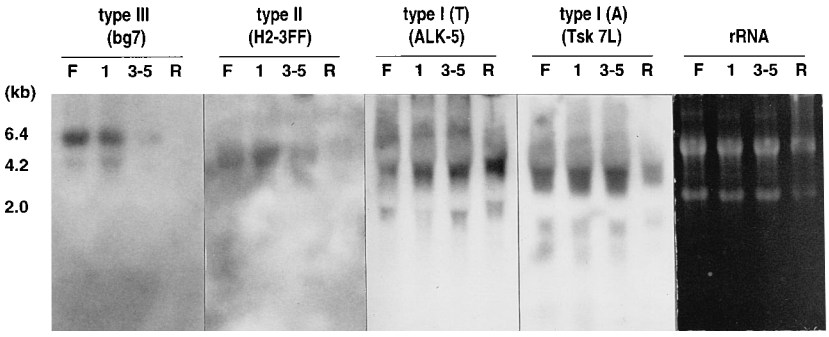

FIG. 3. Steady-state levels of mRNA encoding TGF-β binding sites in fetal rat cell cultures. Twenty-five micrograms of total RNA prepared from serum-deprived NRK-49F fetal rat fibroblasts (lane F) and population 1 (lane 1), population 3-5 (lane 3-5), and ROS 17/2.8 (lane R) cells was fractionated on agarose gels and hybridized with cDNA encoding type III betaglycan (*bg7*), type II TGF-β receptor (*H2-3FF*), type I (T) (for TGF-β) receptor (*Alk-5*), or type I (A) (for activin) receptor (*Tsk 7L*). Four micrograms of each RNA sample was run on a parallel gel to assess rRNA loading and integrity by staining with ethidium bromide. Numbers on the left indicate migration by ethidium bromide-stained RNA standards.

occurred in population 1, while larger increases in alkaline phosphatase activity occurred in population 3-5. Moreover, BMP-2 reduced replication, modestly enhanced collagen synthesis, and potently increased alkaline phosphatase activity in ROS 17/2.8 cultures (Fig. 4 and Table 1). These results suggested that specific effects by BMP-2 also depended on the degree to which cells in each population were presently committed to expression of osteoblast function.

BMP-2 effects on TGF-β binding. BMP-2 appeared to increase osteoblast-like activity incrementally in primary and continuous rat bone-derived cell cultures. Therefore, we examined whether it also altered the TGF-β binding profile on bone cells in ways that were compatible with the intrinsic differences seen in our initial binding studies (Fig. 2). Twenty-four-hour treatment with BMP-2 did not alter TGF-β1 binding in fetal rat fibroblasts. However, it reduced TGF-β binding to betaglycan and type II receptors in population 1 and in population 3-5. In addition, BMP-2 treatment increased relative binding by TGF-β1 at type I receptors in each bone cell culture model. In population 3-5, in which BMP-2 induced the most pronounced and consistent overall shifts in TGF-β binding, its effects were evident at concentrations of 0.3 to 3 nM and between 4 and 24 h of treatment (Fig. 5). To examine the persistence of these changes, population 3-5 cultures were incubated for 48 h with control or BMP-2-supplemented medium or were rinsed and refed with fresh medium without or with BMP-2 during the second 24-h period. As seen in Fig. 6, the inhibitory effects of 24-h treatment with BMP-2 on TGF-β binding to type II and type III sites diminished after 48 h of continuous incubation (lanes 1 and 2), persisted with a second BMP-2 treatment (lanes 3 and 4), and were nearly completely reversed when BMP-2 was omitted from the medium during the second 24-h period (lanes 5 and 6). Nonetheless, the increase in TGF-β binding to type I receptors persisted in cultures that were treated with BMP-2 for the entire 48 h or only during the first 24-h interval.

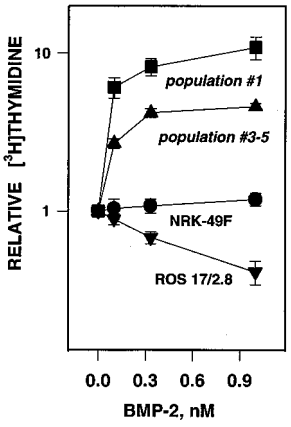

FIG. 4. BMP-2 effects on DNA synthesis in fetal rat cell cultures. Serum-deprived cultures were treated for 23 h with the amounts of BMP-2 shown. DNA synthesis was determined by labeling with [^{3}H]thymidine during the last 2 h of treatment. Data (means ± SE for four replicate samples per condition) are expressed relative to control (no BMP-2) values and on a logarithmic ordinate to demonstrate changes in cultures with endogenously different basal incorporation rates and different types of effects by BMP-2. In the experiments shown, basal [^{3}H]thymidine incorporation (10^3 dpm/0.32 cm^2) was 26.0 ± 1.5 in NRK-49F cultures, 8.1 ± 0.8 in population 1 cultures, 21.5 ± 1.4 in population 3-5 cultures, and 82.2 ± 3.2 in ROS 17/2.8 cultures. Similar effects were found in a minimum of two separate studies. DNA synthesis was significantly increased by BMP-2 levels of 0.1 to 3 nM in population 1 and population 3-5, in which it was maximal at 1 nM, whereas DNA synthesis was significantly decreased at BMP-2 levels above 0.3 nM in ROS 17/2.8 cultures but was unaltered in NRK-49F fetal rat fibroblasts and only minimally increased in primary cultures of fetal rat dermal fibroblasts (not shown).

BMP-2 effects on TGF-β function. The bifunctional effect of BMP-2 on TGF-β1 binding to type I and type II receptors provided an opportunity to assess the relative importance of each site on bone cells. Cultures were treated for 24 h with BMP-2 and then exposed to TGF-β1, duplicating the conditions in Fig. 5, in which changes in TGF-β1 binding occurred. As in Fig. 1, in cultures that were not pretreated with BMP-2, TGF-β1 strongly enhanced DNA synthesis in population 3-5. While some BMP-2 activity persisted during the total treatment period, it dose dependently suppressed the mitogenic effect of TGF-β1. This result was evident at or below maximally effective TGF-β1 concentrations. At concentrations of TGF-β1 on the descending limb of its own biphasic dose-response curve (6, 8–10, 24), BMP-2 had no added effects. BMP-2 also reduced TGF-β activity in population 1, but the overall changes were less pronounced (Fig. 7). In contrast, pretreatment with 0.3 to 3.0 nM BMP-2 did not reduce platelet-derived growth factor AA activity in these cultures, nor did it further reduce the antiproliferative effect of TGF-β1 in ROS 17/2.8 cells (not shown), which exhibit few type II TGF-β receptors (Fig. 2 and 5). Parallel studies to examine if BMP-2 altered other TGF-β actions in bone cells showed no more than additive effects by BMP-2 and TGF-β1 on collagen synthesis in population 1 (Table 1), while as little as 0.3 nM BMP-2 (which reduced binding to type II receptors by more

than 30%) synergistically enhanced TGF-β-induced collagen synthesis in population 3-5 (Fig. 8). Although at high TGF-β levels (0.4 nM) the combined effect of both factors was more nearly additive, pretreatment with BMP-2 (at 0.3 to 3 nM) never decreased the stimulatory effect of TGF-β1 (at 0.004 to 0.4 nM) on collagen synthesis (Fig. 8) or reduced collagen secretion to the medium (not shown) in population 3-5. However, in ROS 17/2.8 cultures, in which the rate of type I collagen synthesis is endogenously high, the combined effect of BMP-2 and TGF-β1 on collagen synthesis again was additive (Table 1). With regard to alkaline phosphatase, enzyme activity was invariably reduced by TGF-β1 in primary bone cell cultures and was increased in ROS 17/2.8 cells (Fig. 1). Nevertheless, pretreatment with BMP-2 sustained an approximate 3.4- to 4-fold increase in enzyme specific activity in population 3-5, even with subsequent exposure to TGF-β1. In contrast, in ROS 17/2.8 cultures, this treatment protocol synergistically enhanced alkaline phosphatase activity (Table 1).

Since TGF-β1 binding to type II receptors reappeared when population 3-5 cells were transiently exposed to BMP-2, changes in TGF-β1 activity were examined within this context. TGF-β-induced DNA synthesis was strongly suppressed when the cells were treated with BMP-2 during the last two 24-h intervals or only during the final 24 h before TGF-β1 addition. However, the mitogenic effect of TGF-β1 was fully evident when cells were exposed to BMP-2 for 24 h and then refed control medium for 24 h prior to TGF-β1 treatment. When collagen synthesis was examined under analogous conditions, synergy with TGF-β1 persisted and increased with continual BMP-2 treatment. If BMP-2 was present only transiently during the first of two 24-h treatment intervals, its ability to enhance TGF-β1 function was diminished (Fig. 9).

DISCUSSION

In vitro and in vivo studies demonstrate that TGF-β has complex and often conflicting effects on skeletal cells. A unifying hypothesis predicts that the function of TGF-β changes as bone cells differentiate, but how this change occurs mechanistically has been difficult to demonstrate (reviewed in reference 7). Our earlier studies showed that cortisol reduced the biological effects of TGF-β in osteoblast-enriched cultures and, in parallel, induced a two- to threefold increase in TGF-β binding at type III sites and a 40 to 60% decrease in TGF-β binding to type I and type II receptors (10). These changes suggested the importance of TGF-β receptor stoichiometry on bone cells but could not assign loss of TGF-β function to increases at type III sites or to decreases at the type II or type I receptor. We also assessed if changes in basal TGF-β activity related to inherent differences in TGF-β binding sites at different states of bone cell differentiation. Initial characterizations revealed less TGF-β binding to type III sites (relative to type I sites) in osteoblast-enriched cultures by comparison with less differentiated fetal rat bone cells (9), and Scatchard analysis demonstrated only a single class of high-affinity binding sites on two highly differentiated fetal rat osteoblast-like cell lines (24). We combined these observations with results from fetal rat fibroblasts and now demonstrate that type I receptors gain prominence as osteoblast-like activity increases. These results also provided an opportunity to examine in vitro if the relative abundance of select cell surface binding sites helped to determine the manner in which bone cells respond to TGF-β treatment. As the amount of TGF-β binding to type III sites decreased, TGF-β was a more effective growth regulator overall. This finding is consistent with the possibility that an overabundance of type III sites may accumulate and conserve cell

TABLE 1. Effects of BMP-2 on TGF-β activity in fetal rat bone cell cultures[a]

Culture	Collagen synthesis (dpm/0.32 cm^2, 10^3)						Alkaline phosphatase activity (pmol/min/μg of protein)					
	Population 1		Population 3-5		ROS 17/2.8		Population 1		Population 3-5		ROS 17/2.8	
	Control	TGF-β	Control	TGF-β	Control	TGF-β	Control	TGF-β	Control	TGF-β	Control	TGF-β
Control	22.6 ± 1.3	50.2 ± 3.1*	24.2 ± 4.4	60.2 ± 3.0*	96.8 ± 3.5	125.0 ± 7.5*	213 ± 16	102 ± 19*	369 ± 13	270 ± 12*	1,872 ± 130	2,560 ± 145**
BMP-2	51.1 ± 2.4*	75.2 ± 7.3*	55.6 ± 3.8*	121.5 ± 8.7**	117.0 ± 6.5*	142.0 ± 6.2*	333 ± 13*	175 ± 5*	1,602 ± 57*	996 ± 39*	3,030 ± 124*	4,818 ± 446**

[a] Serum-deprived cultures were treated with BMP-2 (3 nM) for 24 h and then exposed to a maximum level of TGF-β1 (0.4 nM) for an additional 24-h period. Collagen synthesis was determined by labeling with 12.5 μCi of [^{3}H]proline per ml during the last 2 h of culture. Alkaline phosphatase activity was measured by *p*-nitrophenylphosphate hydrolysis as described previously (35). Data are means ± SE for 6 to 12 replicate samples per condition. Similar effects were found in at least two separate studies. TGF-β1 has dose-related effects on collagen synthesis and alkaline phosphatase in bone cell cultures. These changes are maximal at 0.1 to 0.4 nM (5, 6, 8–10). *, significantly greater than value for untreated cultures, $P < 0.05$; **, significantly greater than increment from TGF-β treatment alone.

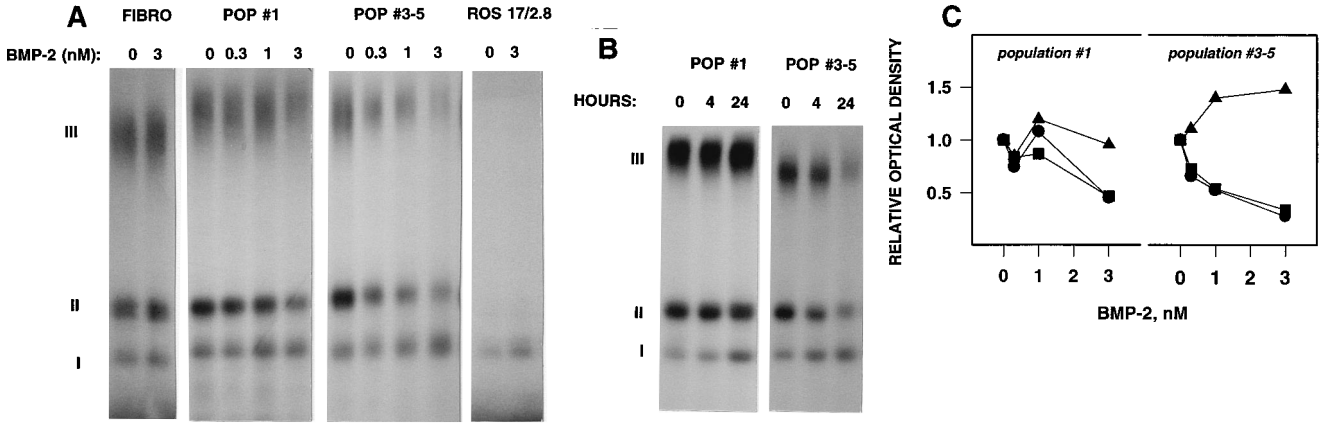

FIG. 5. BMP-2 effects on TGF-β binding in fetal rat bone cells. Extracts from serum-deprived cultures bound and cross-linked to ^{125}I-TGF-β1 were fractionated on 5 to 10% polyacrylamide gels and visualized by fluor-enhanced autoradiography. (A) Continuously cultured NRK-49F fetal rat fibroblasts (FIBRO) and population 1 (POP #1), population 3-5 (POP #3-5), and ROS 17/2.8 cells were treated for 24 h with the amounts of BMP-2 shown. (B) Population 1 and population 3-5 cultures were incubated for 0, 4, or 24 h with 3 nM BMP-2 prior to labeling with ^{125}I-TGF-β1. (C) Densitometry of ^{125}I-TGF-β1 bound to type I (▲), type II (■), and type III (betaglycan) (●) receptors in autoradiographs obtained from population 1 and population 3-5 cultures treated for 24 h with the amounts of BMP-2 indicated. No significant effects by BMP-2 on TGF-β1 binding were ever observed in cultured fibroblasts, whereas similar changes in population 3-5 cultures have been consistently observed. Complexes were designated by analogy to other tissue systems and marked as in the legend to Fig. 2.

surface TGF-β for activation or for later use when a sufficient number of type I or type II signaling receptors are present, as suggested by studies by Lopez-Casillas et al. (33, 34). As the proportion of total binding to type II and type I receptors (relative to type III receptors) increased (and levels of binding to type III and type II receptors were in more equal stoichiometry), TGF-β induced greater mitogenic effects. However, when the type I/type II ratio increased, TGF-β more effectively increased collagen synthesis or alkaline phosphatase activity, features associated with early stages of gene expression during osteoblast differentiation (26, 51).

We also found that BMP-2 drives osteoblast phenotype development, but the cells and processes that it targets are not

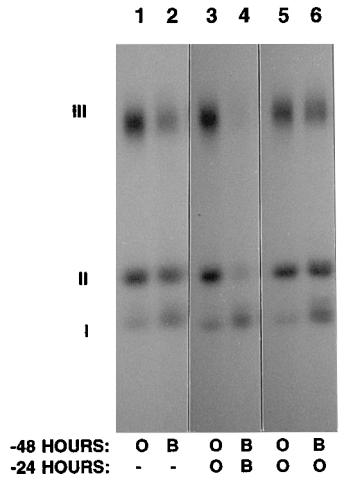

FIG. 6. Transient and persistent effects of BMP-2 on TGF-β binding in population 3-5 osteoblast-enriched cultures from fetal rat bone. Serum-deprived population 3-5 cultures were refed control medium (0) or 3 nM BMP-2 (B). After 24 h, some cultures were left in their original incubation medium (lanes 1 and 2) and some were refed control medium (lanes 3, 5, and 6) or a second dose of BMP-2 (lane 4). After a second 24-h incubation, the cultures were incubated with ^{125}I-TGF-β and cross-linked, and extracts were fractionated on 5 to 10% polyacrylamide gels and visualized by fluor-enhanced autoradiography. Analogous results were obtained in three separate studies. Complexes were designated by analogy to other tissue systems and marked as in the legend to Fig. 2.

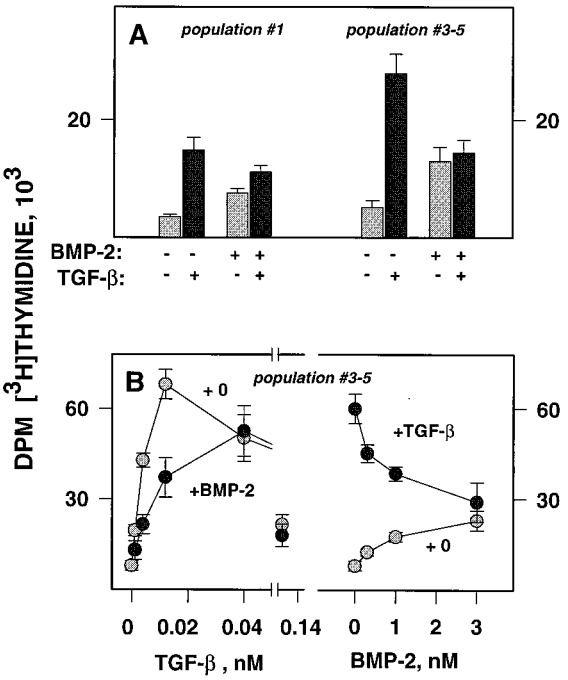

FIG. 7. Effect of TGF-β on DNA synthesis in fetal rat bone cell cultures pretreated with BMP-2. (A) Serum-deprived cultures of population 1 and population 3-5 from fetal rat parietal bone were pretreated with control medium (−) or 3 nM BMP-2 (+) prior to treatment with control medium (−) or 0.012 nM TGF-β1 (+), as indicated. (B) Serum-deprived population 3-5 cultures were pretreated with control medium (0) or 3 nM BMP-2 prior to the addition of the amounts of TGF-β1 shown (left). Alternately, they were pretreated with the amounts of BMP-2 shown prior to treatment with control medium (0) or 0.012 nM TGF-β1 (right). Pretreatments lasted 24 h, and second treatments occurred during the following 23-h interval. Effects of TGF-β on DNA synthesis were determined by labeling with [^{3}H]thymidine during the last 2 h of culture. Data are means ± SE for four to eight replicate samples per condition, and similar effects were found in a minimum of three separate studies. BMP-2 pretreatment at 1 to 3 nM and, by itself, TGF-β1 treatment at all concentrations shown induced significant increases in both cell populations. BMP-2 pretreatment significantly reduced the effect of TGF-β1 at or below 0.012 nM in population 3-5.

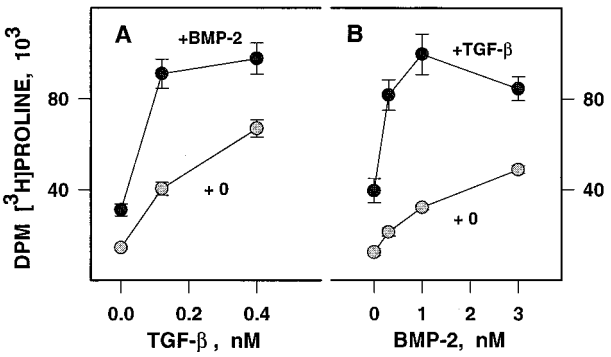

FIG. 8. Synergistic effect of BMP-2 on TGF-β-induced collagen synthesis in osteoblast-enriched fetal rat bone cell cultures. (A) Serum-deprived population 3-5 cultures were pretreated with control medium (0) or 0.3 nM BMP-2 prior to the addition of the amounts of TGF-β1 shown. (B) Alternately, the cultures were pretreated with the amounts of BMP-2 shown prior to treatment with control medium (0) or 0.12 nM TGF-β1. Pretreatments lasted 24 h, and second treatments occurred during the following 24-h interval. Collagen synthesis was determined by labeling with [^{3}H]proline during the last 2 h of culture and by differential sensitivity to purified collagenase. Data are means ± SE for a minimum of six replicate samples per condition, and similar effects were found in a minimum of two separate studies. BMP-2 and TGF-β1 each induced significant increases in collagen synthesis. The amount of activity resulting from the combined effect of both factors was more than additive and was most evident with submaximal concentrations of both factors.

always the same as those influenced by TGF-β treatment (our current studies and references 12 and 53). We now further show that BMP-2 alters the TGF-β binding profile in a way that parallels the native progression seen in population 1, population 3-5, and ROS cells. Importantly, in cells in which BMP-2 decreased TGF-β binding at type II receptors, TGF-β was a less effective mitogen. In cultures in which BMP-2 increased the type I/type II TGF-β receptor ratio, it enhanced TGF-β-induced collagen synthesis or alkaline phosphatase activity; in this context, the effect that was observed depended on the present phenotypic state of the cell being tested. Transient treatment with BMP-2 produced temporary decreases in TGF-β binding to type III and type II sites, while the increase in TGF-β binding to the type I receptor persisted after BMP-2 treatment ceased. However, variations in DNA and collagen synthesis paralleled the loss and recovery of type II receptors, further suggesting that relative, rather than absolute, binding of TGF-β to each site guides its function for osteoblasts. Overall, therefore, recent exposure to BMP-2 alters TGF-β binding and biological activity in ways that seems to prompt bone cells toward the next step in phenotype expression. In addition, our own recent studies (49) indicate that cells at various points within the scheme of mesenchymal cell differentiation are also influenced by BMP-2 in dissimilar ways.

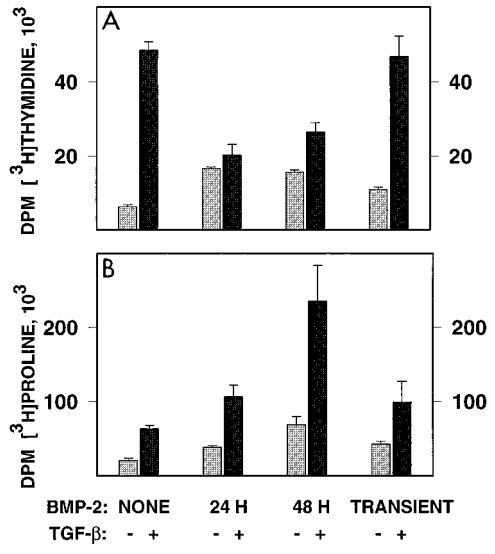

FIG. 9. Transient effects of BMP-2 on TGF-β activity in population 3-5 osteoblast-enriched cultures from fetal rat bone. Serum-deprived population 3-5 cultures were refed control medium (NONE), with 3 nM BMP-2 for 24 or 48 h (repeated at each 24-h interval), or with BMP-2 for 24 h followed by 24 h with control medium (TRANSIENT). Cultures were then refed control medium (−) or 0.012 nM TGF-β1 (for DNA synthesis [A]) or 0.12 nM TGF-β1 (for collagen synthesis [B]) (+) for another 23-h interval. Levels of DNA and collagen synthesis were determined by labeling with [^{3}H]thymidine or [^{3}H]proline during the last 2 h of treatment. Data are means ± SE for six to eight replicate samples per condition, and similar patterns were found in two separate studies.

It is important to note that our current results may be more relevant to early stages of bone development, since we have limited our present data to studies with fetal rat cells. We have done so mainly to restrict possible variations from species differences, maturation status, or virus immortalization. Nonetheless, we have also examined TGF-β binding in several normal and immortalized mouse and human primary, clonal, and osteosarcoma-derived osteoblast-like cell cultures. The relative TGF-β binding profile varied considerably among these cultures. Some cell lines had binding patterns more like that of population 1 cultures, while others had relatively more binding at type I receptors, suggesting that they could represent bone cells at separate stages of differentiation. However, consistent with our present results, BMP treatment tended to increase the proportion of TGF-β binding at type I receptors, although the extent to which decreases occurred at betaglycan and type II receptors depended on their initial relative density in untreated cultures. Using long-term cultures of osteoblast-enriched cells analogous to our population 3-5, several research groups have shown progressive waves in the expression of various bone cell gene products (including early variations in TGF-β1 expression) and the development of three-dimensional bone-like anlages in vitro (reviewed by Stein and Lian [5]). Consequently, the effects of TGF-β on several osteoblast genes (reviewed in reference 7) might be initiated through progressive changes in the TGF-β receptor profile that we now report. Further studies with other in vivo and in vitro models might more precisely assess where receptor variations like those we observe might occur during bone development.

Recent evidence suggests that type II receptors engage TGF-β; the binary complex recruits, phosphorylates, and activates type I receptors; and intracellular events then develop (55). This model originates from studies of mink lung cells chemically mutated to eliminate type II or type I TGF-β receptors and of simian virus 40-transformed monkey kidney fibroblasts (COS-1) that endogenously express few TGF-β receptors. In these systems, endogenous or transfected type I receptors will not engage ligand efficiently without type II receptors, and type II receptors will not signal efficiently without type I receptors (23, 30, 54, 55). Tight coupling among TGF-β, type I, and type II receptors (55) suggests a rigid stoichiometry between the two binding sites and that signaling is limited by the least abundant member of the ternary complex. Some uncertainty in this model might result from the nature of the cells used in its development. The mutated mink lung cells are essentially selected only by their inability to bind or respond to TGF-β (30), and it is difficult to know if mutations in other important genes that limit independent binding or signaling by one or another receptor have not also occurred.

Also, genetic events by which simian virus 40-transformed COS-1 cells normally restrict TGF-β binding site expression could complicate our understanding of the processes occurring in cells that efficiently bind and respond well to TGF-β. In contrast, our present results with ROS 17/2.8 cells, with primary cell cultures from rat bone, and with BMP-2 treatment predict that the amount of TGF-β binding to type I receptors can at times exceed binding to type II receptors and that intracellular signals or events may diverge when one or the other receptor predominates. Consequently, our data do not support a strict codependence between type I and type II receptors for all aspects of TGF-β activity toward skeletal cells. Similarly, Ewton et al. showed an increase in the type I/type II TGF-β receptor binding ratio after myoblasts differentiated to form myotubes, but the authors examined only one aspect of TGF-β function, amino acid uptake, that decreased in parallel with this occurrence (19). Further precedence for independent type I receptor binding is found with other TGF-β/TGF-β receptor gene family members: BMP-2 binding to a type I receptor (termed Brk25D) for the *Drosophila* protein Dpp occurs in the absence of detectable type II receptors and is not enhanced by coexpression of a type II receptor from *Caenorhabditis elegans*, Daf-4, that also binds BMP-2 (46).

In contrast to results in mink lung epithelial cells, in which TGF-β reduces DNA synthesis (30, 55), TGF-β increases bone cell replication prior to terminal differentiation. This effect in bone cells depends on type II receptors but does not eliminate the possibility that type II receptors require type I receptors for signaling. Nonetheless, our results with ROS 17/2.8 cells and with BMP-2-treated cultures suggest that type I receptors may in some instances bind TGF-β and function independently of authentic high-affinity type II sites. Our findings for bone cells could be reconciled with those in which both receptors appear necessary. For example, the type II receptor displays serine/threonine kinase activity independently of ligand engagement, and in this context the type I receptor becomes a suitable substrate for the type II kinase after ligand binding (55). However, no formal evidence exists that TGF-β will or will not bind to previously phosphorylated type I receptors. During differentiation, other kinases may become activated and appropriately phosphorylate type I receptors, enhance TGF-β binding or stabilize the complex, and bypass the need for authentic type II TGF-β receptors. Alternatively, complex formation could occur between type I and type II receptors that individually have greater affinity for different TGF-β gene family members, similar to previous observations (1, 16, 17, 46, 52). Independent downstream events (see, e.g., references 11 and 22) could result from binding only one or the other authentic TGF-β receptor and would in effect eliminate certain events that occur by simultaneous binding to both. In either situation, when bone cells are treated with BMP-2, ligand-bound type II BMP receptors could activate type I TGF-β receptors or act as silent binding site partners. Complicating this is the possibility that type I and type II TGF-β receptors each can form homodimers (27, 56) that might function in only select circumstances or cell types. In further support of our findings, other studies demonstrate that selective or independent effects through type I receptors might also occur in other tissue systems (3, 11). Several possibilities and complications of this complex receptor system have been discussed in a recent review by Derynck (15).

BMP-2 could alter the TGF-β binding profile on bone cells in other ways. Our present results show that decreases in TGF-β binding to type III and type II sites occur in parallel once nearly equivalent binding is achieved at both sites. Initial results show a rapid decrease in type III mRNA but only minimal effects on type II mRNA after BMP-2 treatment, while immunoprecipitation studies with anti-type III antibody invariably show the concomitant presence (in control) or loss (with BMP-2 treatment) of both sites after binding and cross-linking with ^{125}I-TGF-β1 (unpublished results). This finding suggests that type II receptors are less stable when type III receptors are not expressed by bone cells. Decreases at both binding sites are transient unless the cells are continuously exposed to BMP-2, which further implicates the likelihood of posttranscriptional controls. In contrast, the increase in TGF-β binding to type I receptors persists after an initial BMP-2 treatment. Consequently, intricate and independent regulation of all three binding species appears to occur in bone cells. Higher levels of BMP-2 (this report) or longer periods of treatment (unpublished results) will induce similar changes in less differentiated bone cells, suggesting that some effects may depend on the cell type or on developmental stage. We are currently examining changes in transcript and protein stability in control and hormone-treated cultures and the influence of differentiation status in order to clarify these issues further with respect to bone. Nonetheless, our present results demonstrate that complex changes which may reflect the degree of phenotype expression occur with regard to TGF-β binding to bone cells and that these changes can be regulated by morphogens that induce bone formation. In this way, we have been able to discriminate between some events that appear more dependent on individual TGF-β receptors on bone cells. Our results argue for progressive changes in TGF-β receptors and TGF-β function, due in part to effects by BMPs, during osteoblast phenotype expression and continue to emphasize the importance of TGF-β gene family members for skeletal growth.

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