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Activin-A Binding and Biochemical Effects in Osteoblast-Enriched Cultures from Fetal-Rat Parietal Bone

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Activin, a disulfide-linked polypeptide dimer first isolated from gonadal tissue extracts, has amino acid sequence and structural homology with transforming growth factor β (TGFβ). Along with other activities, TGFβ regulates replication and differentiation and interacts with a defined set of binding sites on isolated bone cells. To determine if activin shares these properties, recombinant human activin-A (A-chain homodimer) was examined in osteoblast-enriched cultures obtained from fetal-rat parietal bone. After 23 h of treatment, 60 to 6,000 pM activin-A increased the rate of [^{3}H]thymidine incorporation into DNA 1.5- to 4.0-fold, and at 600 to 6,000 pM, it enhanced the rate of [^{3}H]proline incorporation into collagen and noncollagen protein by up to 1.7-fold. Like earlier studies with TGFβ in primary osteoblast-enriched cultures, the stimulatory effects of activin-A on DNA and protein synthesis were opposed by parathyroid hormone, and the influence of activin-A on collagen synthesis was independent of cell replication. Binding studies with ^{125}I-activin-A indicated approximately 8,000 high-affinity (K_d = 0.4 nM) and 300,000 low-affinity (K_d = 40 to 50 nM) binding sites per cell. Polyacrylamide gel analysis revealed ^{125}I-activin-A-binding complexes of M_r >200,000 and 73,000 which did not appear to correspond to primary TGFβ-binding sites. These results indicate that activin-A produces TGFβ-like effects in bone and that some of these effects may be mediated, at least in part, by distinct activin receptors on bone cells.

Numerous studies indicate that systemic and local (bone-derived) factors alter replication and the expression of differentiated function in cultures of intact bone and isolated bone cells (reviewed in references 4, 9, and 23). Of the many local factors that are produced by bone cells or associated with the extracellular bone matrix, transforming growth factor β (TGFβ) appears to be one of the most abundant and potent growth regulators yet described (9). Bovine bone contains at least two closely related TGFβ isoforms (9, 27, 28) that, along with the prototype human platelet-derived TGFβ and other proteins, are members of a supergene family of growth regulators that are expressed in diverse tissues and species (16, 29, 31, 34, 35).

One of the distally related members of the TGFβ gene family is activin, a polypeptide dimer of M_r 25,000 that contains approximately 30% amino acid sequence homology and a cysteine distribution pattern analogous to that of platelet TGFβ (15, 16). Two activin isoforms have been isolated from porcine follicular extracts. One, termed activin-A, is a homodimer composed of two A subunits. The second, termed activin-AB, is a heterodimer containing two distinct but structurally related A and B activin subunit chains. Each activin subunit is also found as one of the β subunits of inhibin, which is a heterodimer comprising unrelated α (inhibin-specific) and β (activinlike) chains. No differences between the biological effects of activin-A and activin-AB have yet been described. Each activin isoform enhances the release of follicle-stimulating hormone from cultured pituitary cells, whereas basal and activin-stimulated follicle-stimulating hormone release is diminished by inhibin (15, 35). Activin regulates proliferation and differentiation in cultured ovarian, testicular, pituitary, and erythroid precursor cells. Furthermore, the molecule initially termed erythroid differentiation factor (EDF), isolated from cultures of phorbol ester-treated human monoblastic leukemia cells, is essentially identical to activin-A (19). These initial studies indicate a diverse range of autocrine, paracrine, and endocrine activities by activin, many of which also are shared by TGFβ (3, 13–15, 19, 20, 35).

Recent studies have shown that continuously cultured GH_3 pituitary cells contain a TGFβ-binding complex of M_r 70,000 to 74,000 (11) that is distinct from the M_r 65,000, 85,000, and >200,000 complexes observed in bone and many other mesenchymal-tissue-derived cells (8, 11, 26, 33). Radioactive TGFβ on GH_3 cells is displaced by either unlabeled TGFβ or activin, suggesting shared functional activity mediated at the M_r 70,000 to 74,000 binding site in this particular cell type (11). By comparison, EDF binding on murine erythroleukemia cells occurs at M_rs 67,000, 76,000, and 140,000, and EDF is not easily displaced by TGFβ type 1 (TGFβ-1) (14).

There is currently no information on the biochemical effects of activin on bone cells in vivo or in vitro. Therefore, the present studies were initiated to determine if bone is a target tissue for activin, to establish if the effects of activin are similar to those of TGFβ, and to examine if these cells contain activin-binding sites similar to, or distinct from, the TGFβ-binding sites previously observed in this or other culture systems.

MATERIALS AND METHODS

Cell cultures. Parietal bones obtained from 22-day-old rat fetuses were dissected free of sutures and subjected to five sequential collagenase digestions. Although overlap among cells at various stages of osteoblast differentiation is likely, the populations released during the last three collagenase treatments (treatments 3 to 5) are enriched with cells exhibiting osteoblast characteristics (17). Cells from a pool of

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populations 3 to 5 were plated at 12,500 cells per cm^2 in Dulbecco's modified Eagle's medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2), 100 μg of ascorbic acid per ml, penicillin and streptomycin (all from GIBCO), and 10% fetal bovine serum (FBS; Sigma). After reaching confluence (approximately 6×10^4 to 9×10^4 cells per cm^2), the cultures were serum deprived for 20 h. The test agents were then added in serum-free medium, and the cultures were incubated for an additional 23 h. All bone cell experiments were performed with primary cultures. Normal rat kidney fibroblasts (NRK, clone 49F), obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium containing 5% newborn calf serum (GIBCO), penicillin, and streptomycin. Stock NRK-49F cultures were passaged at subconfluence; experiments were performed with confluent cultures that were serum deprived and treated under conditions identical to those for primary bone cells.

Cell replication. The effects of the test agents on DNA synthesis were measured by pulse-labeling cultures with [^{3}H-methyl]thymidine (5 μCi/ml, 80 Ci/mmol; Dupont NEN) during the last 2 h of treatment. [^{3}H]thymidine incorporation into DNA was determined by lysing the cells in 0.1 M sodium dodecyl sulfate–0.1 N sodium hydroxide, collecting the insoluble material formed by precipitation with 10% trichloroacetic acid, and scintillation counting; acid-soluble incorporation in the trichloroacetic acid supernatants was similarly determined. Data were recorded as the total amount of acid-insoluble or acid-soluble [^{3}H]thymidine incorporated per culture well. Cell numbers were determined by counting the cells in an aliquot of a trypsinized cell suspension in a fixed-volume hemacytometer and were expressed as number of cells per square centimeter.

Protein synthesis. To determine the effect of the test agents on collagen and noncollagen protein (NCP) synthesis, cultures were pulsed with 12.5 μCi of [^{3}H-2,3]proline (2.5 Ci/mmol; Dupont NEN) per ml for the last 2 h of treatment. Cell layers were lysed by freeze-thawing; homogenates were collected in 0.5% Triton X-100 (Sigma), precipitated with 10% trichloroacetic acid, and chilled; and the acid-precipitable material was collected by centrifugation. The precipitates were acetone extracted, dried, resolubilized in 0.5 M acetic acid, and neutralized with 0.5 M sodium hydroxide. [^{3}H]proline incorporation into collagenase-digestible protein (CDP) and NCP was measured by using nonspecific protease-free bacterial collagenase (Worthington), as described by Peterkofsky and Diegelmann (22), and shown as the total amount of [^{3}H]proline incorporated per culture. Percent collagen synthesis (PCS) was calculated after correcting for the relative abundance of proline in CDP and NCP. Protein content was determined by the method of Bradford (2).

Binding complex assays. To examine activin-A binding, serum-deprived cultures were incubated in serum-free medium containing 4 mg of bovine serum albumin per ml (binding medium) for 1 h at 37°C and recultured for an additional 1 h in fresh binding medium at 4°C. Activin-A was radiolabeled by the addition of ^{125}I-sodium iodide in the presence of lactoperoxidase, with three additions of hydrogen peroxide at 5-min intervals, and separated from unbound iodine by elution from a C_{18} Sep-Pak (Waters) with 75% acetonitrile in 0.1% trifluoroacetic acid (21). ^{125}I-activin-A (400 Ci/mmol) was lyophilized with 90% recovery, rechromatographed on Sephadex G-75 in 1.0 M acetic acid, and stored at a concentration of 400 nM at 4°C. To examine activin-A binding, ^{125}I-activin-A was then added, without or with unlabeled ligands, for various times at 4°C. Cultures were extracted with 1% Triton X-100, and the amount of bound label was measured in a gamma spectrometer. To visualize activin-A-binding complexes, cultures were serum deprived and preincubated as described above and ^{125}I-activin-A was added for 3 h at 4°C. Cultures were rinsed with chilled binding medium, cross-linked with disuccinimidyl suberate, extracted, and fractionated by electrophoresis on 7.5% polyacrylamide gels, and bound ^{125}I-activin-A was visualized by autoradiography as previously reported (8). ^{125}I-TGFβ (R&D Systems, Inc.; 1,200 Ci/mmol) binding was determined by analogous procedures.

Test agents. Recombinant human activin-A (A-chain homodimer of greater than 99% purity) was produced in a mammalian kidney cell line transfected with a plasmid containing the entire coding region of the β_A-subunit of human inhibin under the control of a cytomegalovirus promoter, as described by Schwall et al. (25) and supplied by Genentech, Inc. Recombinant activin-A was dissolved in 1.0 M acetic acid to obtain a concentration of 5.1 μM and stored at 4°C; this preparation contained less than 0.005% TGFβ when assayed by a radioimmunoassay that detects as little as 0.5 fmol of TGFβ (3a). Recombinant human TGFβ-1 (also supplied by Genentech) was diluted into 0.1 M hydrochloric acid to obtain a concentration of 0.8 μM and stored at 4°C. This preparation has essentially the same activity in osteoblast-enriched cultures from fetal-rat bone as native TGFβ-1 from human or porcine blood platelets (5-9), and all TGFβ isoforms tested to date have similar qualitative effects in this culture model (32). The amino-terminal 34-amino-acid fragment of rat parathyroid hormone (PTH; Sigma), which elicits complete adenylate cyclase-stimulating activity in vitro and PTH-like biochemical effects in isolated bone cell cultures (5, 8, 24), was dissolved in 0.1 M acetic acid–4 mg of bovine serum albumin per ml to obtain a concentration of 100 μM and stored at −80°C. Hydroxyurea (Sigma) was dissolved directly into culture medium immediately prior to use. In control experiments, concentrations of acetic acid and hydrochloric acid equivalent to the levels achieved by the growth factors had no activity in the cell culture assays.

Statistical methods. Data were recorded as means ± standard errors of the means (SE) and were subjected to analysis of variance; statistical differences between groups were assessed by a modified *t* test, with limits set by the Bonferroni inequality equation when multiple concentrations of variables were compared (12).

RESULTS

A 23-h treatment with activin-A increased cell replication in confluent serum-deprived, osteoblast-enriched cultures from fetal-rat parietal bone. This effect was dose related, and at 60 to 6,000 pM (1.5 to 150 ng/ml), activin-A enhanced [^{3}H]thymidine incorporation into DNA by up to 4-fold and cell density by up to 1.5-fold (Table 1). The mitogenic effect of activin-A was relatively transient, however, since DNA synthesis rates fell to 1.9-fold of control rates in cultures treated for 48 h with 6,000 pM activin-A (data not shown). For comparison with other rat cell cultures from nonosseous tissue, the effect of activin-A was examined in NRK-49F rat fibroblasts. In this cell line, 20 to 6,000 pM activin-A did not alter DNA synthesis rates under the same culture and treatment conditions, and only a minimal increase was induced by 400 pM TGFβ. In contrast, 1.25 to 10% FBS enhanced [^{3}H]thymidine incorporation in the NRK-49F cultures by 4- to 25-fold (Fig. 1).

TABLE 1. Effect of activin-A on cell replication in osteoblast-enriched cultures from fetal-rat parietal bone[a]

Activin-A concn (pM)	[^{3}H]thymidine incorporation[b]		No. of cells (10^{-4}/cm^2)[c]
	Acid insoluble	Acid soluble	
0	4.5 ± 0.2 (100 ± 4)	10.1 ± 0.6 (100 ± 6)	6.9 ± 0.2 (100 ± 3)
60	8.0 ± 0.3[d] (178 ± 6)	11.4 ± 0.4 (112 ± 4)	8.5 ± 0.3 (122 ± 6)
600	13.9 ± 1.3[d] (308 ± 28)	13.7 ± 0.3[d] (135 ± 3)	9.6 ± 0.3[d] (139 ± 6)
6,000	19.6 ± 0.9[d] (434 ± 19)	15.3 ± 0.4[d] (151 ± 4)	10.4 ± 0.2[d] (150 ± 3)

[a] Serum-deprived confluent cell cultures were incubated for 23 h without or with human recombinant activin-A. Data are the means ± SE of four replicate 2-cm^2 culture wells per condition. Numbers in parentheses indicate relative effects, where control (no activin-A) = 100.

[b] Trichloracetic acid-insoluble and -soluble [^{3}H]thymidine incorporation (10^{-4} dpm per culture) was measured by pulse-labeling for the last 2 h of culture as described in Materials and Methods.

[c] Determined by counting an aliquot of a trypsinized cell suspension in a fixed-volume hemacytometer.

[d] Significant difference from control ($P < 0.01$).

In the primary osteoblast-enriched cultures used in these studies, recombinant human TGFβ-1 (hereafter referred to as TGFβ) induces a biphasic increase in the rate of DNA synthesis that peaks at approximately 50 to 100 pM TGFβ (5, 8). As shown in Fig. 2, after a 23-h treatment, the stimulatory effect of 60 pM TGFβ was 12- to 14-fold above that of the control, whereas higher TGFβ concentrations (200 and 600 pM) were less stimulatory. In the presence of TGFβ at a submaximal concentration (shown with 6 pM TGFβ in Fig. 2), the combined effect of 200 to 6,000 pM activin-A and TGFβ was additive; at near-maximal TGFβ concentrations (60 pM), the effect of activin-A was biphasic, whereas at higher TGFβ concentrations (600 pM), activin-A did not increase DNA synthesis rates (Fig. 2). Earlier studies revealed that the biochemical effects of TGFβ are opposed by PTH in osteoblast-enriched cell cultures (8); similarly, the mitogenic effect of activin-A, like that of low concentrations of TGFβ, was opposed by 0.2 to 20 nM PTH (Fig. 3).

Activin-A also dose dependently enhanced the rate of collagen synthesis in osteoblast-enriched parietal-bone cell cultures, with an approximate 1.7-fold increase at 6,000 pM (Table 2); the effect of activin-A on NCP synthesis was comparable, such that there were no notably large or reproducible increases in PCS (Tables 2, 3, and 4). Similarly, 23 h of exposure to 6,000 pM activin-A enhanced cellular protein content from a control value of 38.3 ± 3.5 to 51.4 ± 1.5 μg of protein per cm^2 in activin-A-treated cultures ($n = 6$; $P < 0.01$). By comparison, 40 to 400 pM TGFβ enhanced the rate of collagen and NCP synthesis in osteoblast-enriched cultures by 1.8- to 3.6-fold, but the combined effect of TGFβ and 6,000 pM activin-A was no greater than that of TGFβ alone at any concentration examined (Table 3); analogous results were observed with 60 and 600 pM activin-A (data not shown).

The stimulatory influence of activin-A on collagen synthesis (Fig. 4) and NCP synthesis (not shown), like that of

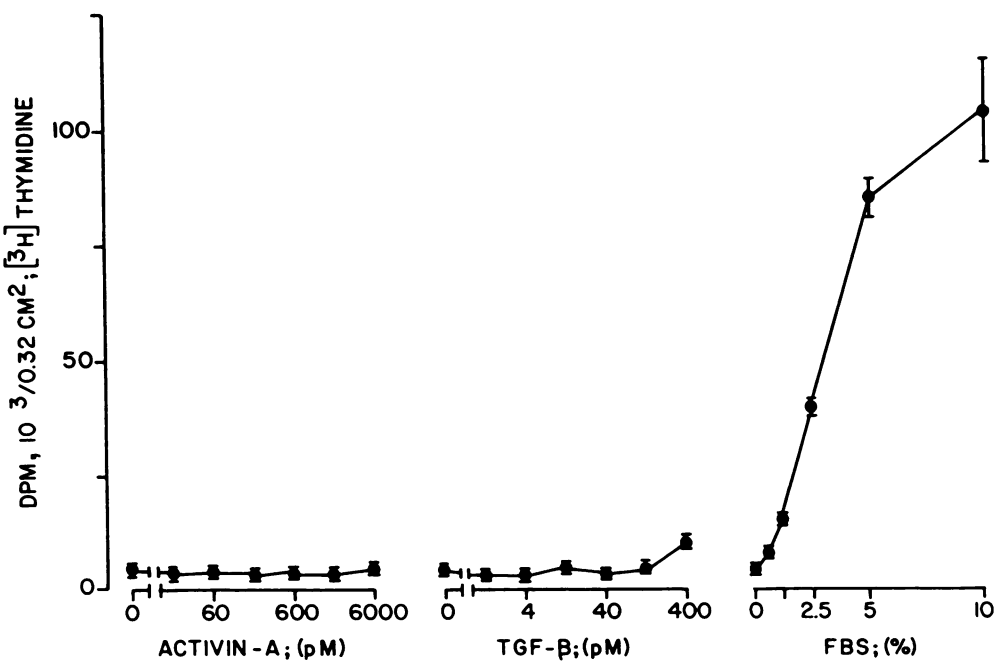

FIG. 1. Relative effects of activin-A, TGFβ-1, and serum on DNA synthesis rates in NRK-49F-cultured fibroblasts. Serum-deprived confluent cell cultures were incubated for 23 h without or with activin-A, TGFβ-1, or FBS at the concentrations shown. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for the last 2 h of culture as described in Materials and Methods; data are the means ± SE of four replicate 0.32-cm^2 culture wells per condition. DNA synthesis was significantly enhanced by TGFβ-1 at 400 pM and by FBS at all concentrations above 1.25% ($P < 0.01$).

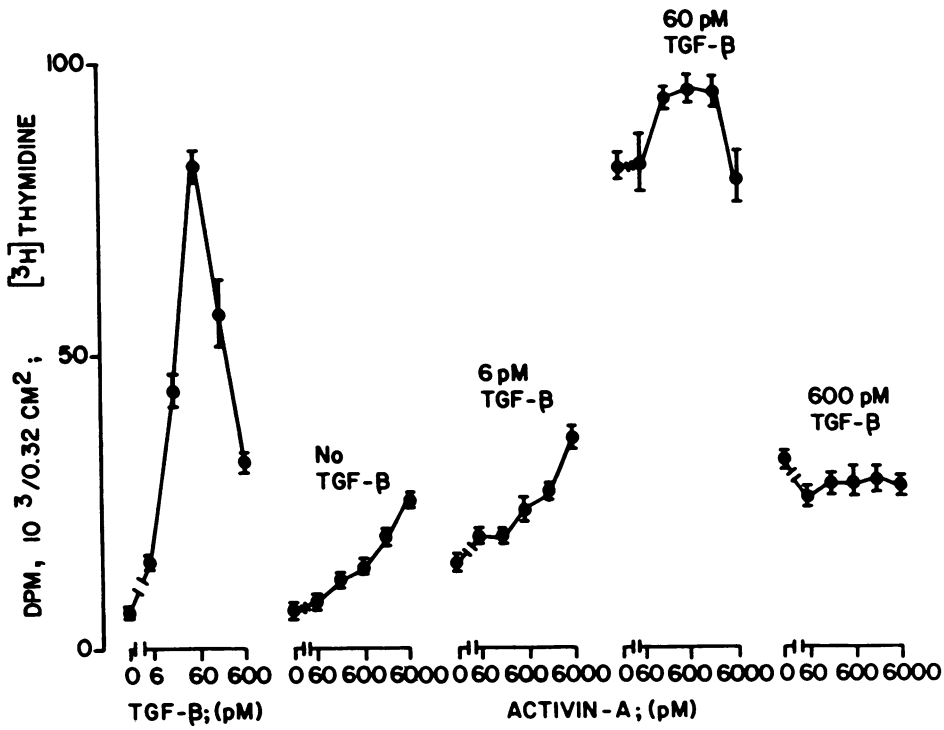

FIG. 2. Independent and combined effects of activin-A and TGFβ-1 on DNA synthesis rates in osteoblast-enriched cultures from fetal-rat parietal bone. Serum-deprived confluent cell cultures were incubated for 23 h without or with TGFβ-1 in the absence or presence of activin-A at the concentrations shown. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for the last 2 h of culture as described in Materials and Methods; data are the means ± SE of four replicate 0.32-cm^2 culture wells per condition. TGFβ-1 at concentrations greater than 6 pM and activin-A at concentrations equal to or greater than 600 pM without or with 6 pM TGFβ-1 significantly enhanced DNA synthesis relative to control (no addition) or TGFβ-1 alone ($P < 0.01$).

TGFβ, was not dependent on its ability to enhance cell replication; increases of similar magnitude occurred in the absence or presence of hydroxyurea, which suppressed DNA synthesis by more than 85% (Fig. 4). Similar to its effect on DNA synthesis, high doses of PTH suppressed protein synthesis and opposed the increase in collagen and NCP synthesis induced by either activin-A (6,000 pM) or a concentration of TGFβ (12 pM) with approximately equivalent biochemical activity (Table 4).

^{125}I-activin-A bound to osteoblast-enriched cell cultures in a time- and dose-dependent manner. Binding was essentially complete after 120 min of incubation at 4°C (Fig. 5A). When these studies were performed with only radiolabeled activin-A, binding approached saturation at concentrations of 4 to 8 nM (not shown), analogous to results in murine erythroleukemia and rat follicular granulosa cell cultures (14, 30). However, when binding was examined by using a combination of radiolabeled and unlabeled activin-A, a relatively higher level in activin-A-binding capacity was observed (Fig. 5B), predicting that the osteoblast-enriched cultures also contained an activin-A-binding site of relatively low affinity. Scatchard analysis was curvilinear, indicating at least two classes of activin-A-binding sites (Fig. 5C). There were approximately 8,000 apparent high-affinity binding sites per cell, with a dissociation constant (K_d) of 0.4 nM, and 300,000 low-affinity binding sites per cell, with a constant (K_d) of 40 to 50 nM.

When the osteoblast-enriched cultures were incubated with a high concentration (2 nM) of ^{125}I-activin-A (to label high- and low-affinity binding sites) and chemically cross-linked, and when the cell extract was then analyzed by polyacrylamide gel electrophoresis and autoradiography, ligand-bound complexes were detected at M_rs of 73,000 and >200,000. Binding was blocked by concentrations of unlabeled activin-A above 3 nM, with a relatively higher degree of displacement at the M_r 73,000 site. Activin-A binding at each location was also reduced by unlabeled TGFβ (Fig. 6 and Table 5).

Extracts from cultures incubated with a lower concentration (0.8 nM) of ^{125}I-activin-A to maximize high-affinity binding site labeling were compared with ^{125}I-TGFβ-labeled cell extracts; the M_r 73,000 ^{125}I-activin-A-labeled complex migrated between the M_r 65,000 and the M_r 85,000 ^{125}I-TGFβ-labeled complexes and was displaced by approximately 50% with a 15-fold molar excess (12 nM) of unlabeled TGFβ. In contrast, even allowing for its 3-fold-higher specific activity, ^{125}I-TGFβ at a 10-fold-lower concentration (0.08 nM) bound to the osteoblast-enriched cultures with a much higher efficiency than ^{125}I-activin; both high- and low-affinity TGFβ-binding sites were 80 to 90% displaced by 10- to 30-fold molar excess (0.8 to 2.4 nM) of unlabeled TGFβ (Fig. 7).

DISCUSSION

The present studies demonstrate that concentrations of activin-A that are known to regulate biochemical effects in gonadal, pituitary, and hematological tissue (3, 13, 35) induce dose-related increases in replication and protein synthesis in osteoblast-enriched cultures from fetal-rat parietal

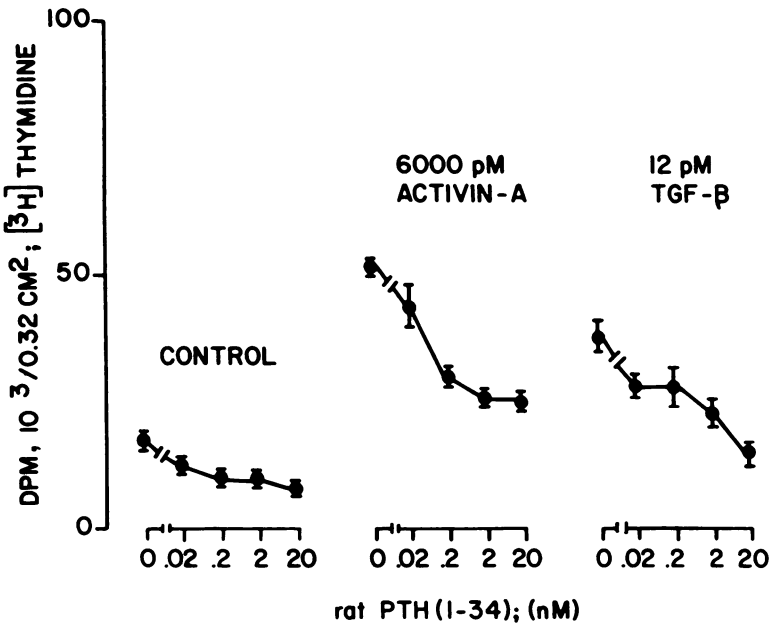

FIG. 3. Effect of PTH on DNA synthesis stimulated by activin-A or TGFβ-1 in osteoblast-enriched cultures from fetal-rat parietal bone. Serum-deprived confluent cell cultures were incubated for 23 h without or with PTH in the absence or presence of TGFβ-1 or activin-A at the concentrations shown. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for the last 2 h of culture as described in Materials and Methods; data are the means ± SE of four replicate 0.32-cm^2 culture wells per condition. PTH at 0.2 to 20 nM significantly decreased basal and activin-A- or TGFβ-1-stimulated DNA synthesis ($P < 0.05$).

bone. Like TGFβ, activin-A fails to be a potent mitogen in confluent NRK-49F fibroblast cultures. Nevertheless, activin-A is approximately 500-fold less potent than TGFβ in the osteoblast-enriched cultures. These relative effects are not surprising since, on a molar basis, TGFβ is also 10- to 100-fold more potent than other well-described growth promoters (epidermal growth factor, basic and acidic fibroblast growth factors, and various platelet-derived growth factor isoforms) in this tissue system (7, 9, 10, 18). In contrast to the apparent additive effects of activin-A and TGFβ on DNA synthesis, which appear to follow the same biphasic dose-response curve induced by TGFβ alone, the effects of the combination on protein synthesis are more complex. Even at a submaximal TGFβ level (with regard to its effect on protein synthesis), the combined effects of TGFβ and a stimulatory concentration of activin-A are no greater than the effect of TGFβ alone. This observation suggests that the effects of activin-A and TGFβ on protein synthesis may be generated through independent binding sites or by way of separate, mutually exclusive, secondary intracellular signals. Regardless, as was found with earlier studies with TGFβ (6, 8), the stimulatory influence of activin-A on protein synthesis is independent of cell replication and is opposed by PTH, suggesting a common regulatory pathway in this process independent of the stimulatory signal.

Cross-linking studies with radiolabeled activin-A demonstrated two classes of binding sites migrating at M_rs 73,000 and >200,000 on polyacrylamide gels in osteoblast-enriched cultures from fetal-rat bone. Earlier studies with GH_3 pitu-

TABLE 2. Effect of activin-A on collagen and NCP synthesis in osteoblast-enriched cultures obtained from fetal-rat parietal bone[a]

Activin-A concn (pM)	Level of:		PCS
	CDP[b]	NCP[b]	
0	17.2 ± 0.5	49.4 ± 2.3	6.1 ± 0.2
60	19.1 ± 0.6	46.5 ± 1.2	7.0 ± 0.2[c]
600	23.3 ± 2.0[c]	57.1 ± 4.4	6.9 ± 0.1
6,000	29.0 ± 0.4[c]	64.8 ± 1.3[c]	7.5 ± 0.4[c]

[a] Serum-deprived confluent cultures were incubated for 23 h without or with activin-A at the concentrations shown. Cultures were pulse-labeled with [^{3}H]proline for the last 2 h of culture as described in Materials and Methods.
[b] Data are shown as 10^{-3} dpm/2-cm^2 culture and are the means ± SE of five or six replicate culture wells per condition.
[c] Significant difference from control (no activin-A) ($P < 0.02$).

TABLE 3. Combined effects of activin-A and TGFβ-1 on collagen and NCP synthesis in osteoblast-enriched cultures obtained from fetal-rat parietal bone[a]

Concn (pM)		Level of:		PCS
Activin-A	TGFβ-1	CDP[b]	NCP[b]	
0	0	23.3 ± 1.3	39.8 ± 0.8	9.8 ± 0.4
0	40	41.9 ± 1.3[c]	67.2 ± 0.8[c]	10.3 ± 0.2
0	120	56.4 ± 2.7[c]	86.0 ± 4.2[c]	10.9 ± 0.3
0	400	83.9 ± 4.9[c]	118.6 ± 4.5[c]	11.6 ± 0.3[c]
6,000	0	35.1 ± 1.6[c]	57.0 ± 1.6[c]	9.8 ± 0.5
6,000	40	42.5 ± 2.0[c]	67.6 ± 3.2[c,d]	10.3 ± 0.2
6,000	120	58.3 ± 2.2[c,d]	82.4 ± 1.2[c,d]	11.6 ± 0.3[c,d]
6,000	400	82.6 ± 2.6[c,d]	125.4 ± 2.3[c,d]	11.1 ± 0.3[c]

[a] Serum-deprived confluent cultures were incubated for 23 h without or with activin-A or TGFβ-1 at the concentrations shown. Cultures were pulse-labeled with [^{3}H]proline for the last 2 h of culture as described in Materials and Methods.
[b] Data are shown as 10^{-3} dpm/2-cm^2 culture and are the means ± SE of five or six replicate culture wells per condition.
[c] Significant difference from control (no addition) ($P < 0.01$).
[d] Significant difference from activin-A alone ($P < 0.05$).

TABLE 4. Opposing effects of PTH and activin-A or TGFβ-1 on collagen and NCP synthesis in osteoblast-enriched cultures from fetal-rat parietal bone[a]

Addition (concn)	Level of:		PCS
	CDP[b]	NCP[b]	
Nothing	21.2 ± 1.0	54.7 ± 2.0	6.7 ± 0.2
Activin-A (6,000 pM)	28.4 ± 0.9[c]	69.6 ± 2.4[c]	7.1 ± 0.1
TGFβ-1 (12 pM)	30.2 ± 1.0[c]	74.4 ± 1.1[c]	7.0 ± 0.1
PTH (20 nM)	12.6 ± 0.3[c]	45.5 ± 0.9[c]	4.6 ± 0.1[c]
PTH + activin-A	19.6 ± 1.4[d]	57.3 ± 2.4[d]	5.9 ± 0.2[c,d]
PTH + TGFβ-1	19.9 ± 0.4[d]	57.6 ± 1.5[d]	6.0 ± 0.1[c,d]

[a] Serum-deprived confluent cultures were incubated for 23 h without or with PTH, activin-A, or TGFβ-1 at the concentrations shown. Cultures were pulse-labeled with [^{3}H]proline for the last 2 h of culture as described in Materials and Methods.

[b] Data are shown as 10^{-3} dpm/2-cm^2 culture and are the means ± SE of six replicate culture wells per condition.

[c] Significant difference from control (no addition) ($P < 0.02$).

[d] Significant difference from PTH alone and from the same concentration of factor in the absence of PTH ($P < 0.05$).

itary cells demonstrated a previously undescribed membrane-associated TGFβ-binding complex of M_r 70,000 to 74,000 whose labeling was displaced by unlabeled activin-A (11). This complex was comparable to the M_r 73,000 ^{125}I-activin-A-binding complex in osteoblast-enriched cultures. The present studies using ^{125}I-activin-A and isolated bone cells also produce results similar to those with ^{125}I-EDF (now shown to be activin-A; 19) in murine erythroleukemia cell cultures (14). Although the erythroleukemia cells also contain more than a single activin-A–EDF-binding complex when analyzed by cross-linking and polyacrylamide gel analysis, they possess one complex migrating at an M_r of 76,000 (14) that may be analogous to the M_r 73,000 complex found in fetal-rat osteoblast cultures. Therefore, in at least these three (erythroid, pituitary, and bone) tissues, there may be a common binding complex at M_r 70,000 to 76,000 that represents the primary signal transducing receptor for activin-A. Furthermore, both the blood and bone cell cultures contain several thousand high-affinity activin-A–EDF-binding sites per cell with a K_d of 0.3 to 0.4 nM (this report; 14). ^{125}I-activin-A may bind more specifically within the M_r 73,000 complex by relation to the M_r >200,000 activin-binding complex also found in bone cells, since binding and displacement by lower concentrations of radiolabeled and

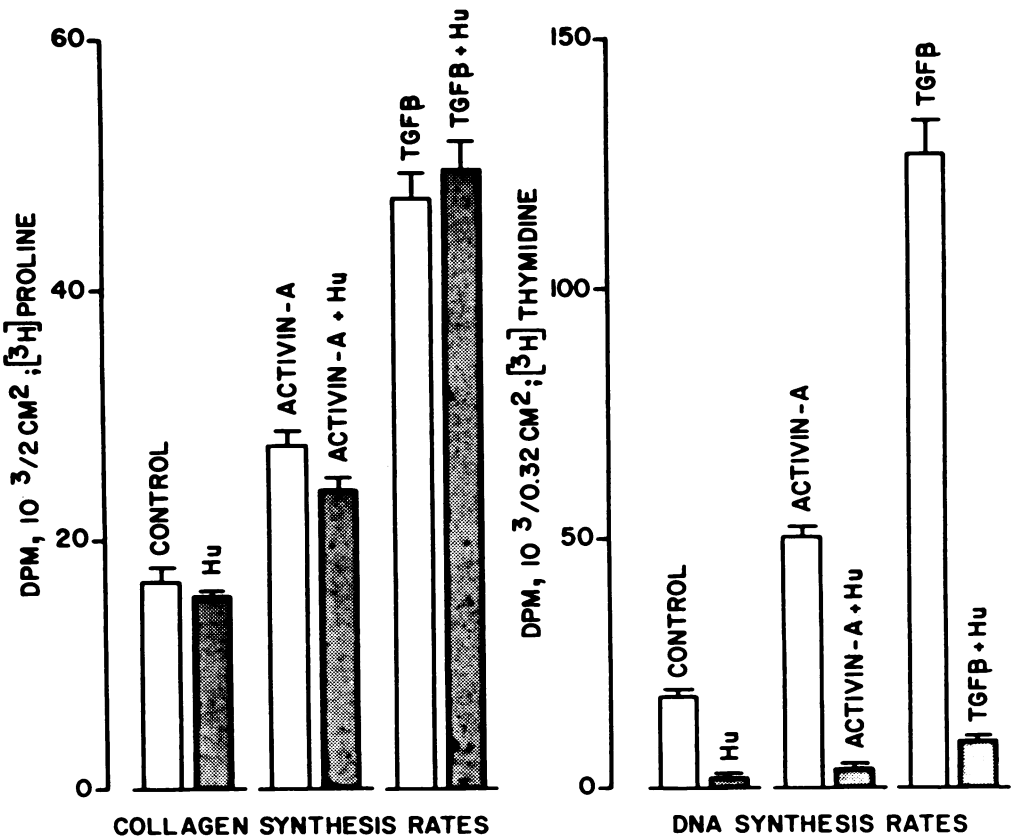

FIG. 4. Effects of activin-A and TGFβ-1 on collagen synthesis in growth-inhibited osteoblast-enriched cultures from fetal-rat parietal bone. Serum-deprived confluent cell cultures were incubated for 23 h without or with 1 mM hydroxyurea (Hu) in the absence or presence of 2,000 pM activin-A or 120 pM TGFβ-1. Collagen synthesis (left panel) was measured by pulse-labeling with [^{3}H]proline for the last 2 h of culture and analyzed by the CDP assay described in Materials and Methods. Data are the means ± SE of six replicate 2-cm^2 culture wells per condition. Activin-A and TGFβ-1 each significantly enhanced collagen synthesis rates in the absence or presence of hydroxyurea ($P < 0.01$), but the effect of each agent was not significantly affected by hydroxyurea. DNA synthesis was measured by pulse-labeling with [^{3}H]thymidine for the last 2 h of culture as described in the text; data are the means ± SE of four replicate 0.32-cm^2 culture wells per condition. DNA synthesis was enhanced by activin-A or TGFβ-1, and the effect of each agent was significantly inhibited by hydroxyurea ($P < 0.01$).

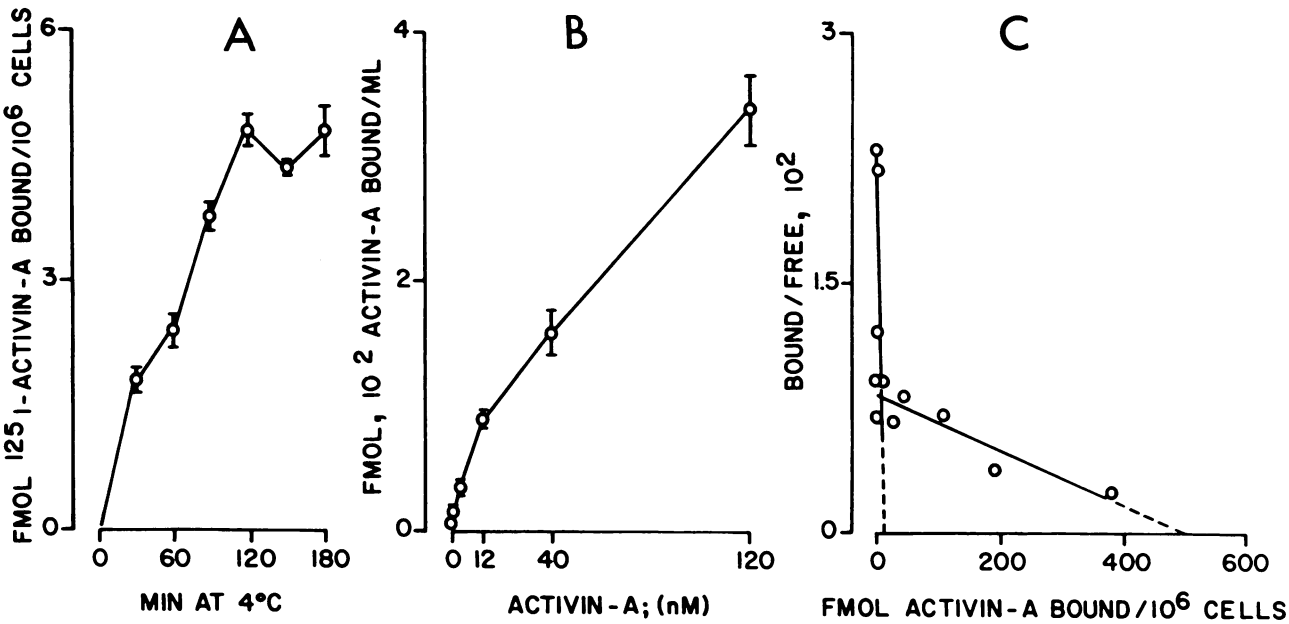

FIG. 5. Activin-A binding to osteoblast-enriched cultures from fetal-rat parietal bone. Serum-deprived confluent cultures were rinsed, preincubated, labeled with ^{125}I-activin-A (400 Ci/mmol), and extracted as described in Materials and Methods. Nonspecific binding (35% of total binding), determined by incubation with a 1,000-fold molar excess of unlabeled activin-A, was subtracted to calculate specific binding. (A) Cultures were incubated with 0.4 nM ^{125}I-activin-A for the times shown. Each datum point is the average of three replicate samples. (B and C) Cultures were incubated with 0.04 to 4 nM ^{125}I-activin-A in the absence or presence of 0.4 to 120 nM unlabeled activin-A (to span the range of high- and low-affinity activin-A-binding sites) for 3 h at 4°C. The molar amount of activin-A bound was calculated after corrections were made for differences in specific activity in each group. Each datum point is the average of three replicate determinations. Data in panel B are shown as a function of the total molar concentration of activin-A included in the binding assay. Data in panel C are shown as a function of the amount of activin-A bound per 10^6 cells; each line was determined by linear regression analysis of all datum points consistent with a correlation coefficient (r) greater than or equal to 0.92.

unlabeled activin-A occur more efficiently at the M_r 73,000 site. As happened in competition studies with GH_3 cells, unlabeled TGFβ may also bind to the M_r 73,000 complex in bone cell cultures, since at high concentrations it can displace radiolabeled-activin-A binding at this location.

These studies further demonstrate that the M_r 73,000 activin-A-binding complex coexists within the same cultures that also bind ^{125}I-TGFβ at M_r 65,000, 85,000, and >200,000. In contrast, negligible binding by low levels of ^{125}I-TGFβ occurs at the M_r 73,000 complex in the osteoblast-enriched cell culture system, indicating that binding at the other three locations is more specific for TGFβ by bone cells. Alternately, TGFβ binding within the M_r 65,000, 85,000 or >200,000 complexes could produce steric or allosteric alterations that preclude its binding at M_r 73,000, and this effect would not be observed in cultures, such as of GH_3 cells, lacking the first three binding sites. The relationship between the M_r >200,000 complexes that contain ^{125}I-activin-A and those that contain ^{125}I-TGFβ is unknown. Some small degree of cross competition at the M_r >200,000 binding site may be possible, however, since activin-A at 60 to 6,000 pM decreases ^{125}I-TGFβ binding by 5 to 20% at this position in isolated bone cell cultures, whereas unlabeled TGFβ at 240 and 2,400 pM decreases ^{125}I-TGFβ binding at this site by 62 and 87% (4a). No biochemical function has been convincingly ascribed to TGFβ ligand-receptor complexes of M_r >200,000 (1, 26), and binding at this location may represent an extracellular storage site for some growth regulators by connective tissue cells.

The present experiments cannot distinguish whether ra-

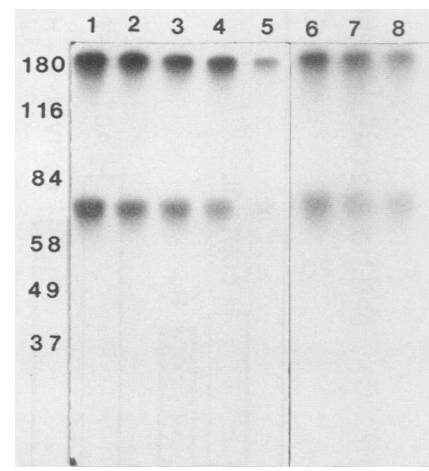

FIG. 6. Activin-A-binding complexes in osteoblast-enriched cultures from fetal-rat parietal bone. Serum-deprived confluent 9.6-cm^2 cultures were incubated for 3 h with 2 nM ^{125}I-activin-A (400 Ci/mmol) without or with unlabeled activin-A or TGFβ-1. At the end of incubation, the cultures were rinsed and cross-linked with disuccinimidyl suberate and fractionated by electrophoresis through a 7.5% polyacrylamide gel. ^{125}I-labeled binding complexes were displayed by autoradiography as described in Materials and Methods. Lane 1, No addition. Lanes 2 through 5: 3, 10, 30, and 100 nM activin-A. Lanes 6 through 8: 0.5, 1.5, and 5 nM TGFβ-1. Numbers on the left show positions of migration by standards with the indicated M_r (10^{-3}).

TABLE 5. Relative displacement of ^{125}I-activin-A by unlabeled activin-A or TGFβ-1 in osteoblast-enriched cultures from fetal-rat parietal bone[a]

Addition (nM)	10^{-3} cpm bound[b]	OD_{525}, relative area[c]	
		M_r >200,000	M_r 73,000
Nothing	25.1 (0.8)	1.00	1.00
Activin-A			
3	23.3 (0.1)	1.00	0.82
10	18.7 (2.2)	0.90	0.75
30	16.1 (1.6)	0.78	0.68
100	11.6 (0.6)	0.52	0.40
TGFβ-1			
0.5	24.0 (1.8)	1.00	1.06
1.5	23.6 (1.1)	0.92	0.80
5	19.8 (1.7)	0.80	0.70

[a] Serum-deprived confluent 9.6-cm^2 cultures were incubated for 3 h with 2 nM ^{125}I-activin-A (400 Ci/mmol) without or with unlabeled activin-A or TGFβ-1, cross-linked, and fractionated by gel electrophoresis, and ^{125}I-labeled complexes were displayed by autoradiography.

[b] Determined in a gamma spectrometer prior to electrophoresis. Data are the average of two cultures, and numbers in parentheses are ranges of both values.

[c] Areas of exposure of the bands migrating at M_rs >200,000 and 73,000 (Fig. 7) were measured by densitometry. Illumination was at 525 nm, and values are optical density (OD) relative to that when no unlabeled ligand was added.

diolabeled activin-A and TGFβ bind to the same cell or to cells of different phenotypes or maturational stages within the cultures. These studies also cannot determine whether the biochemical effects of activin-A in osteoblast-enriched cultures occur by way of only one of the activin-A-binding sites that we noted or by some combination of both. Significant effects are observed with lower activin-A levels that are below the K_d for high-affinity binding, but relatively high concentrations (which exceed the calculated capacity for higher-affinity activin-binding sites in these cultures) do not maximally stimulate DNA or protein synthesis; therefore, the effects of activin-A may be through both high- and low-affinity binding complexes.

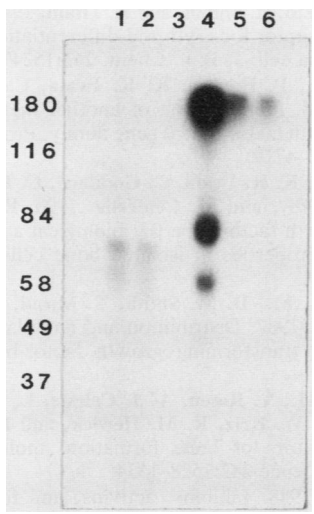

FIG. 7. Comparison of activin-A- and TGFβ-1-binding complexes in osteoblast-enriched cultures from fetal-rat parietal bone. Serum-deprived confluent 4.8-cm^2 cultures were labeled with 0.8 nM ^{125}I-activin-A (400 Ci/mmol) or 0.08 nM ^{125}I-TGFβ-1 (1,200 Ci/mmol) without or with unlabeled TGFβ-1, cross-linked, fractionated by polyacrylamide gel electrophoresis, and displayed by autoradiography as described in the legend to Fig. 6. ^{125}I-activin-A was used with no addition (lane 1) or 40 nM TGFβ-1 (lane 2). ^{125}I-TGFβ-1 was used with no addition (lane 4), 0.80 nM TGFβ-1 (lane 5), or 2.4 nM TGFβ-1 (lane 6). Lane 3 was blank. Numbers on the left show positions of migration by standards with the indicated M_r (10^{-3}).

Similar to TGFβ (9, 29), activin-A also has complex positive and negative effects on cell proliferation and expression of differentiated cell function that are specific to individual cell types. For example, activin-A increases erythroid precursor cell proliferation and accounts for the activity first termed EDF (3, 19) but decreases proliferation by cultured ovarian and testicular cells (13). Furthermore, activin-A, like TGFβ, enhances follicle-stimulating hormone release by cultured pituitary cells (15, 35) but inhibits steroid production in testicular cell cultures (13). Not all biochemical activities are shared by these two factors, however, since TGFβ does not appear to enhance erythroid precursor differentiation (20). These and the present studies with bone cells therefore suggest that some, but not all, of the effects of activin-A and TGFβ may be mediated through overlapping binding sites.

These are the first studies that describe the biochemical activity of activin-A in skeletal-tissue-derived cells and suggest that activin-A may regulate bone cell activity during development; however, these effects might also predict alterations in bone cell function in response to hyperplastic activin-A secretion from gonadal or blood cell tumors. Nevertheless, recent studies indicate that in addition to the several TGFβ isoforms isolated from platelets and bone (27–29), other members of the TGFβ-related family, termed bone morphogenetic proteins (34), may be involved in particular stages of bone formation or repair. Further investigations will be necessary to determine if any of these TGFβ-like proteins exert their effects through one or more of the TGFβ- or activin-binding complexes or whether the activin-binding complex is used by other growth regulators in bone tissue.

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