Functional Regulation of Osteoblastic Cells by the Interaction of Activin-A with Follistatin*

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Bone homeostasis is controlled chiefly by the interaction of two different kinds of cell types, osteoblasts and osteoclasts. The former cells, which are supposed to be derived from the stromal cells, participate in bone formation; the latter cells, derived from hematomonocytic cells, are active in bone resorption. Besides hormonal control, the involvement of various cytokines has been shown to be indispensable to the coordinated action of these cells. These include transforming growth factor (TGF)−β family peptides, which are involved in divergent aspects of cell differentiation (1). Many authors have studied extensively the biological role of TGF-β1 in bone tissues. Osteoblastic cells, which proliferation themselves by an autocrine mechanism (2) and enhance the production of extracellular matrix such as collagen, secrete TGF-β1 (3). TGF-β1 has an inhibitory effect on the differentiation of osteoclast cells (4). Because both TGF-β1 and TGF-β2 are proved to be abundant in bone matrix tissues as well as in platelets (5, 6), they may actually play a significant role in vivo. Bone morphogenetic proteins (7), known as potent cartilage inducers in ectopic tissues, are also shown to be involved in the regulation of osteoblastic cells (8, 9).

Activin-A, a member of the TGF-β family, was originally isolated from ovarian fluids as a stimulator of follicle-stimulating hormone secretion from pituitary gland (10). Recent analyses not only confirmed the identity of activin-A with erythroid differentiation factor (11, 12) but also disclosed diverse biological roles (13), including mesoderm-inducing activity (14−16) and regulation of bone differentiation (17, 18). Follistatin, an inhibitor of follicle-stimulating hormone secretion from the pituitary gland (19), was shown to be an activin-binding protein (20), and the interaction of activin-A with follistatin is also presumed to have a significant role in extraosseous tissues (21−24). However, little is known about the role of activin-A and follistatin in bone tissues. Recently, Centrella et al. (25) reported that activin-A executes TGF-β-like effects through its own binding sites in osteoblast-enriched cultures from rat parietal bone.

Here, we investigated the effect of activin-A on osteoblastic cells using an MC3T3-E1 cell line that is established from newborn mouse calvaria and is capable of differentiating into cells that produce calcified bone matrix in vitro (26). We observed a specific binding of 125I-activin-A on MC3T3-E1 cells, a mitogenic effect of activin-A on cell replication, and an inhibitory effect of activin-A on the expression of alkaline phosphatase. These unique effects of activin-A are dynamically regulated by follistatin, whose expression may be developmentally regulated in osteoblastic differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—A murine osteoblastic cell line (MC3T3-E1), a human osteosarcoma cell line (HOS), and a murine Friend erythroleukemia cell line (F5-5) were maintained in an alpha modification of Eagle’s minimum essential medium (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratories, Cleveland) and 10 μg/ml gentamicin sulfate (Schering-Plough, Osaka, Japan) at 37 °C in a humidified atmosphere of 5% CO2.

Materials—Recombinant activin-A produced from Chinese hamster ovary cells (12) was a kind gift from Drs. Eto and Shibai (Central Research Laboratories, Ajinomoto Co., Kawasaki, Japan). Porcine follistatin (20) was a generous gift from Dr. Sugino (Institute of Physical and Chemical Research, Saitama, Japan). Na235S and [3H] thymidine were purchased from Du Pont-New England Nuclear.

Determination of 125I-Activin-A Binding—Lodination of activin-A was performed by the chloramine-T method as described elsewhere (27). The specific activity of 125I-activin-A ranged from 10,000 to 25,000 cpm/ng. Binding experiments were performed under cell-attached conditions according to the method of Massague and Liker (28) with some modifications. Briefly, MC3T3-E1 cells were plated on 24-well multiplates (10 cells in 500 μl of α-MEM + 10% FBS) and incubated for 4 days to a confluent state. Cells were then washed twice with Ca2+-free phosphate-buffered saline and resuspended in α-MEM containing 25 mM HEPES, pH 7.5, and 2 mg/ml bovine serum albumin (binding buffer) for 1 h. Cells were washed once and incubated with stepwise concentrations of 125I-activin-A in 500 μl of binding buffer at 37 °C for 1 h. A 200-fold excess amount of unlabeled activin-A was added to some samples to determine the nonspecific binding of 125I-activin-A. Incubation was terminated by
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The plate reader (SLT, Salzburg, Austria) was used to determine the standard curve of p-nitrophenol. A typical saturation binding curve was obtained (Fig. 1), with an apparent Kd of 260 PM. Follistatin purified from porcine ovaries and a-MEM containing 10% FBS were added as control samples before the [3H]thymidine uptake. Statistical differences were assessed by Duncan’s multiple range test.

RESULTS

Demonstration of 125I-Activin-A Binding to MC3T3-E1 Cells—When MC3T3-E1 cells were challenged with 125I-activin-A, a typical saturation binding curve was obtained (Fig. 1, inset). Scatchard plot analysis demonstrated that MC3T3-E1 cells have a single class of binding sites (5,600 sites/cell), with an apparent Kd of 260 PM.

The Effect of Activin-A on the DNA Synthesis—The effect of activin-A on the DNA synthesis of MC3T3-E1 cells was different under various conditions of cells (Fig. 2). For the sparse cells the mitogenic effect was remarkable under 0.3% FBS (Fig. 2a). A 3-fold increase of [3H]thymidine uptake was observed with as low as 0.1 nM activin-A. However, no significant effects were observed under 10% FBS (Fig. 2b). Cells proliferate so fast under 10% FBS that the effect of activin-A may be masked. As for the cells in a confluent state, the mitogenic effect was observed both under 0.3% (Fig. 2c) and 10% FBS (Fig. 2d) although the effective concentration was more than 10 nM. When cells were induced to differentiate by treatment with 1 µM RA under 0.3% FBS for 2 days (Fig.

The se'nse primer, 5-GTCGAGGATCCCTGGGAACTGCTGGCT-3' (the 688-712 position and an HindIII site), was combined with the antisense primer, 5-CCTAGAAGCTTGCCCAGGCA-3' (500 ng of total RNA essentially according to Gubler and Hoffman (31). To avoid false-positive results by the contamination of genomic DNA, samples with or without treatment of avian myeloblastosis virus reverse transcriptase (Seikagaku Kogyo Co., Tokyo) were prepared simultaneously. One-tenth of the resulting cDNA was used as template for PCR. Oligonucleotide primers were designed on the basis of the DNA sequence of human follistatin (32). The sense primer, 5-GTCGAGGATCCCTGGGAACTGCTGGCT-CCGTCAAACG-3 (the 113-137 oligonucleotide position of human follistatin and an extra BamHI restriction enzyme site), was combined with the antisense primer, 5-CTAGAAGCTTGCCCAGGCA-GGTAGCCTTTTC-3 (the 688-712 position and an HindIII site), in the PCR reaction to generate a putative 622-base pair cDNA fragment. The temperature program for the amplification was 30 cycles of 1 min at 94 °C, 2 min at 55 °C, and 5 min at 72 °C. The products were separated on a 1% agarose gel and visualized with ethidium bromide. The band of the corresponding size was digested with BamHI and HindIII, cloned into the pUC18 plasmid (Takara), and sequenced by the dyeideoxy method (33).

Ligand Blot Analysis of Follistatin in the Conditioned Medium of MC3T3-E1 Cells—Ligand blotting was done as described previously (20) with minor modifications. MC3T3-E1 cells were incubated (1 × 10^6 cells in 10 ml of a-MEM + 10% FBS/10-cm^2 cultured dish) for 4 days to a confluent state, and then cells were induced to differentiate under 0.3% FBS and 1 µM RA. The conditioned medium (20 ml) was collected every 2 days, changing to a fresh medium. They were added with sulfa-Cellulofine (Seikagaku Kogyo) and stirred gently for 12 h. The gels were washed with 0.35 M NaCl, 20 mM Tris-HCl, pH 7.5, twice, washed with distilled water once, and eluted with 1% SDS, 100 mM Tris-HCl, pH 7.5. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (12%) and electrophobotted onto an Immobilon membrane (Millipore) with the use of a semidyfer filter system. After incubation in 10% skim milk overnight, the blots were treated with 100 ng/ml 125I-activin-A in the presence or absence of a 100-fold excess of unlabeled activin-A in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5 (TBS) containing 0.1% Triton X-100 for 2 h at room temperature. 500 ng of follistatin purified from porcine ovaries and 20 ml of a-MEM containing 10% FBS were also blotted as positive and negative controls, respectively. They were washed with TBS containing 0.1% Triton X-100 for 1 h, and the autoradiograph was taken by 48-h exposure at -80 °C with an intensifying screen.

Statistical Methods—Data are expressed as mean ± S.E. Statistical differences were assessed by Duncan’s multiple range test.

FIG. 1. Northern blot analysis of MC3T3-E1 cells probed with mouse follistatin—6 µg of poly(A)+ RNA was extracted from MC3T3-E1 cells and from mouse uterus. They were electrophoresed on 1.0 agarose under denaturing conditions, blotted to nylon membrane, and hybridized with a mouse follistatin probe which was prepared as mentioned above. Washing conditions were 0.2 × SSC, 0.1% SDS at 65 °C for 30 min, and the autoradiograph was taken by 96-h exposure at -80 °C with an intensifying screen.

The se'nse primer, 5-GTCGAGGATCCCTGGGAACTGCTGGCT-3' (the 688-712 position and an HindIII site), was combined with the antisense primer, 5-CCTAGAAGCTTGCCCAGGCA-GGTAGCCTTTTC-3' (the 688-712 position and an HindIII site), in the PCR reaction to generate a putative 622-base pair cDNA fragment. The temperature program for the amplification was 30 cycles of 1 min at 94 °C, 2 min at 55 °C, and 5 min at 72 °C. The products were separated on a 1% agarose gel and visualized with ethidium bromide. The band of the corresponding size was digested with BamHI and HindIII, cloned into the pUC18 plasmid (Takara), and sequenced by the dyeideoxy method (33).

Reverse Transcription-Phosphatase Assay—The measurement of alkaline phosphatase activity was done according to the method of Gray et al. (29) with minor modifications. Briefly, MC3T3-E1 cells were incubated in 48-well plates (2 × 10^6 cells in 500 µl of α-MEM containing 10% FBS/well) for 4 days to a confluent state. The cells were washed once and incubated with a-MEM + 10% FBS + 100 ng/ml activin-A, a-MEM containing various volumes of conditioned medium (RA), activin-A, porcine follistatin, and their combinations. After 48 h the cells were washed with phosphate-buffered saline twice and added to 200 µl of 0.56 M 2-amino-2-methyl-1-propanol, 1 mM MgCl_2, and 10 mM Na_2 p-nitrophenyl phosphate. At the same time, cell-free wells containing 200 µl of α-MEM with various concentrations of p-nitrophenol were prepared. They were incubated at 37 °C for 1 h and added to 200 µl of NaOH. After 100 µl of each supernatant was transferred to a 96-well plate, the absorbance of p-nitrophenol in a cell-free well or formed by the conversion of p-nitrophenyl phosphate was determined at 405 nm in an enzyme immunoassay. The standard curve was constructed from the absorbance values plotted against the nmol of p-nitrophenol cell-free well. The enzymatic activity of each sample was assessed from the standard curve and expressed as nmol of p-nitrophenol produced per 10^6 cells during the 1-h incubation.

Determination of Anti-activin-A Activity in the Conditioned Medium of Cells—The anti-activin-A activity was evaluated as an inhibitory activity on F5-5 cells which were induced to differentiate by activin-A (11, 12). MC3T3-E1 and HOS cells were incubated (1 × 10^6 cells in 10 ml of α-MEM + 10% FBS/10-cm^2 cultured dish) for 4 days to a confluent state with no medium change, and the conditioned medium containing freshly from each dish (5-6 cells or 20 ml of 10% well) were plated in 96-well microtiter plates in the presence of 5 ng/ml activin-A with various volumes of the conditioned medium or α-MEM containing 10% FBS with or without 100 ng/ml porcine follistatin. α-MEM containing 10% FBS was added so that the final volume became 200 µl. After 5 days the degree of erythroid differentiation was determined by microscopically counting dianisidin-staining positive cells (12).

Cell numbers were counted for some control samples before the [3H]thymidine treatment.

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removal of the medium and cells were washed three times with ice-cold binding buffer. Bound radioactivity was measured after the cells were solubilized with 1% Triton X-100, 125 mM NaCl, and 2 mg/ml bovine serum albumin. Scatchard analysis was performed as described previously (20).
to porcine follistatin, and the anti-activin-A activity was detected by treatment with 5 ng/ml activin-A for 5 days. Additionally, the conditioned media of MC3T3-E1 cells and HOS cells was measured. As shown in Fig. 2, the [3H]thymidine uptake at a lower concentration but increased at higher concentrations, showing a biphasic dose-response pattern.

Inhibitory Effect of Activin-A on Alkaline Phosphatase Expression—RA significantly induced alkaline phosphatase activity of MC3T3-E1 cells in a dose-dependent manner as described previously (34) (Fig. 3a). On the contrary, activin-A, by itself (Fig. 3b), showed a remarkable inhibitory effect. Although porcine follistatin had little effect by itself, it abolished the inhibitory activity of activin-A in a dose-dependent manner (Fig. 3, d and e).

Anti-activin-A Activity in the Conditioned Medium of MC3T3-E1 and HOS Cells—Anti-activin-A activity was identified in the conditioned media of MC3T3-E1 cells and HOS cells. As shown in Fig. 4, about 60% F5-5 cells were differentiated by treatment with 5 ng/ml activin-A for 5 days. Addition of the conditioned medium of MC3T3-E1 cells dose-dependently inhibited the differentiation in a manner similar to porcine follistatin, and the anti-activin-A activity was estimated to be approximately 5 ng/ml porcine follistatin. The anti-activin-A activity was also detectable in the conditioned medium of HOS cells although it was a few times less than that of MC3T3-E1 cells.

The Expression of Follistatin mRNA in Both MC3T3-E1 Cells and HOS Cells—The expression of follistatin mRNA was demonstrated in both MC3T3-E1 cells and HOS cells by RT-PCR. A PCR product with the corresponding length (622 base pairs) was obtained in samples treated with avian myeloblastosis virus reverse transcriptase (Fig. 5a). The subcloning and sequencing confirmed it to be the cDNA of mouse follistatin. A partial amino acid sequence deduced from the cDNA obtained from MC3T3-E1 cells was about 98% homologous to the corresponding region (9-191 amino acids) of the human sequence (Fig. 5b). In case of HOS cells, the sequence was identical to that of human follistatin (data not shown).

The Production of Follistatin in the Conditioned Medium of MC3T3-E1 Cells—Ligand blot analysis with $^{125}$I-activin-A
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Fig. 4. Anti-activin-A activities in the conditioned medium of MC3T3-1 and HOS cells. F5-5 cells (2 × 10^5 cells/well) were induced to differentiate by 5 ng/ml activin-A. The conditioned medium prepared from either MC3T3-E1 cells (●) or HOS cells (□) and α-MEM containing 10% FBS with (A) or without (O) the addition of 100 ng/ml porcine follistatin was added to the F5-5 cultures by stepwise dilution. After 5 days the degree of F5-5 cell differentiation was measured by counting dianisidine-positive cells. Each point is the mean of two samples. Similar results were obtained in two independent experiments.

Fig. 5. Detection of follistatin mRNA by RT-PCR and Northern blotting analysis. Details are described under "Experimental Procedures." The corresponding length of peptides, is known to exert divergent effects on the proliferation and differentiation of various cells (13). In Balb/C 3T3 cells, activin-A is proved to show a unique dual effect on the cell cycle (37); it has a competence-inducing activity as well as inhibiting the progression-inducing activity of platelet-poor plasma. Thus, activin-A seems to exert divergent effects according to the states of responding cells. Taking this into

DISCUSSION

During the course of investigating 125I-activin-A binding to various cultured cell lines, we found that MC3T3-E1 cells contain a high number of activin-A binding sites (5,600 sites/cell) (Fig. 1). Most cultured cells expressed activin-A binding sites ubiquitously but in low numbers as compared with that of F5-5 cells (3,500 sites/cell) or embryonal carcinoma cell lines (i.e. P19 cells; 5200 sites/cell) (34). The binding constant of 125I-activin-A to MC3T3-E1 cells (Kd = 0.26 nM) also corresponds to those of the high affinity binding sites of F5-5 and P19 cells (35). Activin-A is capable of inducing F5-5 cells to differentiate into hemoglobin-synthesizing cells (11, 12) and inhibiting the differentiation of P19 cells to neural cells (17, 36). Therefore, we suspected that activin-A may have a significant function on MC3T3-E1 cells.
were treated with RA in the confluent state, [3H]thymidine addition of RA (Fig. 3a) (34). Interestingly, the effect of cell density, serum concentration and treatment of RA (Fig. 2). The mitogenic effect of activin-A was predominant when MC3T3-E1 cells were in an undifferentiated stage, regardless of the degree of confluence. MC3T3-E1 cells are known to differentiate spontaneously after reaching a confluent state, with the differentiation process being accelerated by low serum and several reagents including RA (34). When cells were treated with RA in the confluent state, [3H]thymidine uptake was remarkably decreased, and the dose-response curve of the activin-A effect showed a biphasic pattern. These observations are, for the most part, in agreement with those reported recently by Centrella et al. (25). Using primary cultured osteoblasts from rat parietal bone, they observed 8,000 high affinity binding sites for 125I-activin-A as well as a mitogenic activity.

Alkaline phosphatase is one of markers of osteogenic cell differentiation and is also known to be stimulated by the addition of RA (Fig. 3a) (34). Interestingly, the effect of activin-A was competitive to RA. Activin-A by itself or even in the presence of RA, inhibited the expression of alkaline phosphatase (Fig. 3a, b and c). The inhibitory effect on this differentiation marker could be the result of the mitogenic activity of activin-A.

Although we observed some unique effects of activin-A on MC3T3-E1 cells, the effects seem to be more or less weaker than those of other cytokines such as TGF-β (38) or those of activin-A itself on other tissues (11, 14, 17, 18). In [3H]thymidine incorporation experiments, the mitogenic effect was not simply proportional to the concentration of activin-A but was indicative of the threshold of the concentration, which was also unstable according to the conditions of cells (Fig. 2). One possible explanation for this may be an endogenous production of other factors that modify the action of activin-A, leading to a more complicated pattern of the regulation of MC3T3-E1 cells.

We have detected the presence of an anti-activin-A activity in the conditioned medium of MC3T3-E1 cells (Fig. 1). Recent studies of follistatin transcripts by in situ hybridization (21, 23), RNase protection (22), and Northern blotting (23, 24) suggested the involvement of follistatin in the regulation of activin-A action in various tissues. Here, we first detected the production of follistatin mRNA by RT-PCR in both MC3T3-E1 cells and HOS cells (Fig. 5, a and b). Using the 622-base pair cDNA fragment, the presence of follistatin mRNA is also demonstrated by Northern blotting, suggesting that a relatively large amount of follistatin mRNA is transcribed in MC3T3-E1 cells (Fig. 5c). We next confirmed the production of follistatin at the protein level. Two forms of follistatin (32 and 35 kDa) are produced by alternative splicing (32), and they are modified further with glycosylation (19, 20). The pattern of bands derived from the conditioned medium of MC3T3-E1 cells is similar to that of pituitary, in which the 35-kDa band is more predominant (39). These results suggest that follistatin is being produced and secreted by these cells and probably is responsible for the anti-activin-A activity. More interestingly, the expression of follistatin was down-regulated when the cells were treated with RA (Fig. 6b). As for activin-A, neither Northern blotting nor bioassay as erythroid differentiation factor detected the expression of activin-A by these cells (data not shown). Presumably these cells produce follistatin according to the condition of cells, which interact with exogenously derived activin.

We found previously that activin-A is mitogenic on P19 cells (17). It has an inhibitory effect on the differentiation of P19 cells and some neuroblastoma cell lines (17, 36). Furthermore, the expression of follistatin is predominant when P19 cells are in an undifferentiated stage. It should be noted that the observations obtained in MC3T3-E1 cells are similar to those in P19 cells. Similar mechanisms may operate in these two different cell types. An in situ hybridization study in ovarian tissues has also indicated that the follistatin mRNA is abundant in secondary or tertiary follicles when granulosa cells seem to be in a rapid growth phase (21). The expression of follistatin may be regulated developmentally during a specific stage of differentiation, especially while cells are in rapid proliferation.

Activin-A has been shown recently to be involved in the morphogenesis of the early development in the amphibian system (14-16). Our results suggest that activin-A effects are predominant when cells are in a growing phase. Activin-A can regulate not only the cell number but also the degree of differentiation of the cells. The activin-A action seems to be threshold dependent. Then, how is follistatin involved in the regulation of activin-A action? We could observe little effect of follistatin on MC3T3-E1 cells. Follistatin only antagonized the stimulatory effect of activin-A on the secretion of alkaline phosphatase (Fig. 3, d and e). In the pituitary, follistatin was shown to inhibit follicle-stimulating hormone secretion by neutralizing the stimulatory effect of activin-A, and the indirect effect was quite different from the direct action of inhibin (39). These results may indicate that follistatin is expressed to inhibit the mitogenic activity of activin-A in P19 or MC3T3-E1 cells. However, we cannot rule out other possibilities. Follistatin may serve as a carrier of activin to the receptor because the affinity of the binding of activin to follistatin is a little weaker than that of activin to its receptor (20). The complex of activin-A and follistatin also may have some other unknown functions. Further investigations are required before the exact role of follistatin is established. Moreover, the roles of other activin-A-related proteins, such as activin-AB, activin-B or inhibins, are also obscure. In the ovary, these proteins appear to participate cooperatively in the proliferation and differentiation of follicles during development. The mechanism employed in the ovarian follicle may be operating in other steps of cell differentiation including neurogenesis and osteogenesis.

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