Journal of Cellular Biochemistry 75:206–214 (1999)

Inhibitory Effects of Activin-A on Osteoblast Differentiation During Cultures of Fetal Rat Calvarial Cells

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Abstract Activin-A is a member of the transforming growth factor-β (TGF-β) superfamily and is expressed by osteoblasts. However, the role of activin-A on osteoblasts is not clearly understood. We examined the effects of activin-A on osteoblast proliferation or differentiation, and mineralization by the osteoblasts in the first subcultures of fetal rat osteoblasts obtained from calvarial bones. Exogenous activin-A led to impaired formation of bone nodules in a dose-dependent manner, although it did not influence cell proliferation using an MTT assay. This inhibitory effect depended upon the time at which activin-A was added to the culture media, and the effect was most significant when addition took place at the early phase of the culture. In addition, exogenous activin-A inhibited gene expression of type I procollagen, alkaline phosphatase, osteonectin, and osteopontin in the cultured cells using Northern blot analysis. The peak of osteocalcin mRNA was delayed. Gene expression for TGF-β was not influenced by exogenous activin-A. The βA subunit (activin-A) mRNA was detected during the early phase of this culture. These results indicate that activin-A inhibited early differentiation of the fetal rat calvarial cells, or osteoblasts. J. Cell. Biochem. 75:206–214, 1999.

Key words: activin-A; osteoblast differentiation; bone nodule; type I collagen; alkaline phosphatase; osteonectin; osteopontin; osteocalcin

Activins were originally isolated from gonadal fluids as a stimulator of follicle-stimulating hormone (FSH) secretion from the anterior pituitary grand [Ling et al., 1986; Vale et al., 1986]. Activins are homodimers or heterodimers of inhibin βA or βB subunits and are members of a transforming growth factor-β (TGF-β) superfamily. Activin-A has also been isolated as a factor named erythroid differentiation factor (EDF) from the conditioned medium of a phorbol ester-stimulated human monocytic cell line by virtue of its hemoglobin synthesis activity in a mouse Friend erythroleukemic cell line [Murata et al., 1988]. It was later found that activin-A has a sequence identical to that of EDF. Recent studies have shown that activin-A is recognized not only as a regulator for FSH secretion but also as a cytokine or growth factor with important roles to play in cellular growth and differentiation, mesoderm-inducing activity [Smith et al., 1990; van den Eijnden-Van Raaij et al., 1990], and regulation of neural differentiation [Hashimoto et al., 1990]. Activin-A (βA homodimer) is the only activin known to be present in bone and cartilage [Ogawa et al., 1992]. Activin-A also demonstrates a potent activity in bone formation. Activin-A induces an increase in replication and protein synthesis in osteoblastic cells [Centrella et al., 1991; Hashimoto et al., 1992] and enhances ectopic bone formation induced by bone morphogenetic protein (BMP) [Ogawa et al., 1992]. In addition, a subperiosteal injection of activin-A induces ectopic bone formation in rats [Oue et al., 1994]. Activin receptors, type I (ActRI), type II (ActRII), and type IIB (ActRIIB) receptor, are expressed by osteoblasts during early intramembranous and endochondral bone formation in rat fracture calls [Shuto et al., 1997]. The βA subunit knockout mice have a cleft palate and an absence of incisors [Matzuk et al., 1995b], while a small percentage of Ac-

Grant sponsor: Ministry of Health and Welfare's Research Grant for Specific Diseases, Japan.

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Received 7 January 1999; Accepted 13 April 1999

tRII knock-out mice show mandibular hypoplasia and other facial and skeletal abnormalities [Matzuk et al., 1995]. Furthermore, activin and follistatin (an activin-binding protein) are reported to regulate endochondral bone formation [Funaba et al., 1996]. These studies indicate that activin-A may play an important role in bone metabolism. However, the role of activin-A in the differentiation of osteoblasts or mineralization is not clearly understood.

This article examines the effects of activin-A on osteoblast differentiation and mineralization in the culture system of fetal rat calvarial (FRC) cells, and the role of activin-A in osteogenesis is discussed.

MATERIALS AND METHODS

Recombinant human activin-A was a generous gift from Y. Eto (Ajinomoto Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan).

Cell Culture

Calvaria from 21-day-old fetal Sprague-Dawley rats were isolated and subjected to sequential digestions lasting 20, 20, and 90 min at 37°C in 1 mg/ml collagenase P (Behringer-Mannheim, Indianapolis, IN)/0.25% trypsin (Gibco, Grand Island, NY), as previously described [Aronow et al., 1990; Bellows et al., 1986]. Cells from the first two digests were discarded, but cells released from the third digests were then resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics. The cells were plated at 6,500 cells/cm^2 on plastic dishes. At confluence (set as day 0), the time course of mineralization was accelerated by the addition of BGJb medium (Gibco) supplemented with 10% FBS, 50 μg/ml ascorbic acid (Gibco), and 10 mM β-glycerol phosphate (Sigma Chemical Co., St. Louis, MO).

Cells were incubated during the culture with 10, 50, or 100 ng/ml of human recombinant activin-A on days 0–5, 5–9, 9–14, or 14–19. Control cells were incubated with just the vehicle (0.07 M sodium acetate) instead of human recombinant activin-A in each group.

This experiment was reviewed by the Committee of the Ethics on Animal Experiment in Faculty of Medicine, Kyushu University, and carried out under the control of the Guideline for Animal Experiment in Faculty of Medicine, Kyushu University, and The Law (No.105) and Notification (No. 6) of the government.

MTT Assay

This assay is dependent on the reduction of MTT (Wako, Tokyo, Japan) by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically [Mosmann, 1983].

Cells were plated on 24-well plates (Falcon 3047; Beckton Dickinson Labware, NJ). After reaching confluence, 100 μl MTT (5 mg/ml) in phosphate-buffered saline (PBS) was added to each culture well, followed by incubation at 37°C for 4 h. The medium was then aspirated from the well as completely as possible without disturbing the formazan crystals or cells on the plastic surface; 1 ml acid isopropanol (0.04 N HCl in isopropanol) was then added to each well. The plates were then agitated on a plate shaker for 5 min, and 100 μl of this liquid were transferred to 96-well microplates. The optical density was recorded at 570–650 nm on the plate reader (NJ-2001; Nulge Nunc International, Tokyo, Japan). This process was demonstrated every 2 or 3 days after confluence. In all experiments, three replicate wells were used to determine each point.

Bone Nodule Assay

For quantitation of bone nodule formation, cells were plated on 12-well plates (Falcon 3043). The plates were rinsed twice in ice-cold phosphate-buffered saline (PBS) and fixed for 30 min in absolute methanol (−20°C) and stained by the von Kossa technique (30 min in 3% $AgNO_3$) on days 0, 5, 9, 14, and 19. Nodules were assessed with respect to number, total nodule area, and mean nodule size, using NIH image software on a computer. In all experiments, three replicate wells were used to determine each point.

RNA Preparation and Northern Blot Analysis

Primary cultures, prepared as described above on 6-well dishes (Falcon 3046), were washed twice with ice-cold PBS, and total cellular RNA was isolated by the acid guanidium-thiocyanate-phenol-chloroform extraction method on days 0, 5, 9, 14, and 19. Briefly, washed cells were incubated in 1 vol denaturing solution (4 M guanidine thiocyanate and 25 mM sodium citrate, pH 7.0), 0.1 vol of 2 M

sodium acetate, pH 4.0, 1 vol phenol, and 0.2 vol chloroform. RNA was then precipitated with isopropanol, washed with ethanol, and resuspended in diethylpyrocarbonate (DEPC)-treated water. Total cellular RNA concentration was quantified by ultraviolet (UV) absorption at 260 nm.

For Northern blot analysis, equal amounts of RNA samples were fractionated by electrophoresis in 1% agarose gel containing 2.2 M formaldehyde, before being stained with ethidium bromide to confirm the integrity and loading amount of RNA in each lane. RNA was transferred to nylon membranes (Nytran; Shleicher & Schuell, Keene, NH) using a standard method. After transfer, the RNA was UV crosslinked to the membrane (Funakoshi, Tokyo, Japan) and dried. cDNA probes were labeled with ^{32}P by a random priming kit (Prime-It RmT; Stratagene, San Diego, CA). Before hybridization, these probes were purified using a column (NucTrap probe purification columns; Stratagene). Prehybridization was carried out at 68°C in hybridization solution (Quick Hyb; Stratagene). After 1-h prehybridization, the DNA probe was added to the hybridization solution with 150 mg of denatured salmon sperm DNA. Hybridization was carried out for 12–16 h at 68°C. The membranes were then washed twice with 2-fold SSC (1-fold SSC = 0.15 NaCl, 0.015 M Na citrate) and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min each, and then once with 0.1-fold SSC and 0.1% SDS at 60°C for 30 min After the membranes were dried at room temperature, the radioactivity of the hybridized probe was analyzed using a Bio-image analyzer BAS 2000 (Fuji Photo Film, Tokyo, Japan).

Rat pro-α_1(I) chain of type I collagen cDNA probe was a gift from Dr. D. Rowe (University of Connecticut, Farmington, CT). The rat alkaline phosphatase probe was a gift from Drs. G. Rodan and M. Thiede (Merck, Sharp and Dohme, West Point, PA). The cDNA probe for osteonectin was a gift from Dr. M.E. Bolander (Mayo Clinic, Rochester, MN). The rat osteopontin probe was kindly provided by Dr. K. Yoon (Merck, Sharp and Dohme). The cDNA probe for rat osteocalcin (bone gla protein) was kindly provided by Dr. J. Wozney (Genetics Institute, Cambridge, MA, USA). Human TGF-β1 cDNA probe was a gift from Dr. G.I. Bell (Chiron Corp., Emeryville, CA). The human inhibin βa subunit cDNA probe was kindly provided by Dr M. Muramatsu (Saitama Medical School, Saitama, Japan).

Statistical Analysis

All results are expressed as the mean ±SEM. Statistical significance was estimated using either the unpaired two-sample *t*-test or a one-way analysis of variance (ANOVA).

RESULTS

In the control groups, MTT activity of the FRC cells began to increase on day 2 and continued to increase until day 9 or 11. After that, no further significant changes in MTT activity were seen. Treatment of activin-A did not influence the MTT activity (Fig. 1).

FRC cells began to form multilayered nodules on day 5 in the control cultures. They were mineralized by day 19. The size of the bone nodules increased after day 9. Activin-A treatment on days 0–5 resulted in a significant decrease of bone nodule formation (Fig. 2). The number of bone nodules was decreased by activin-A treatment on days 0–5 in a dose-dependent manner (Fig. 3A). These inhibitory effects of activin-A treatment were reduced when the treatment was on days 5–9 or on days 9–14. For the treatment on days 14–19, there were no significant differences between the activin-A treatment groups and the control groups. The effects of activin-A treatment on the total area and the average area of bone nodules were similar to those on the number of bone nodules (Fig. 3B,C). Treatment of activin-A had the effect of suppressing bone nodule formation, especially during the early phase of culture.

In order to investigate the effects of activin-A on differentiation of the FRC cells, gene expression of various extracellular matrix proteins acting as osteoblast differentiation markers were determined including type I (α1) procollagen (Col I), alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OP), and osteocalcin (OC) in the culture treated with 100 ng/ml of activin-A on days 0–5 (Figs. 4, 5). Col I mRNA was highly expressed during the culture of FRC cells, and the levels peaked on day 5 in the control groups. Activin-A treatment suppressed the expression of Col I on days 5, 9, and 14. ALP mRNA levels peaked on days 5 and 9 in controls. Activin-A treatment suppressed the level of ALP mRNA on day 5. ON and OP mRNA

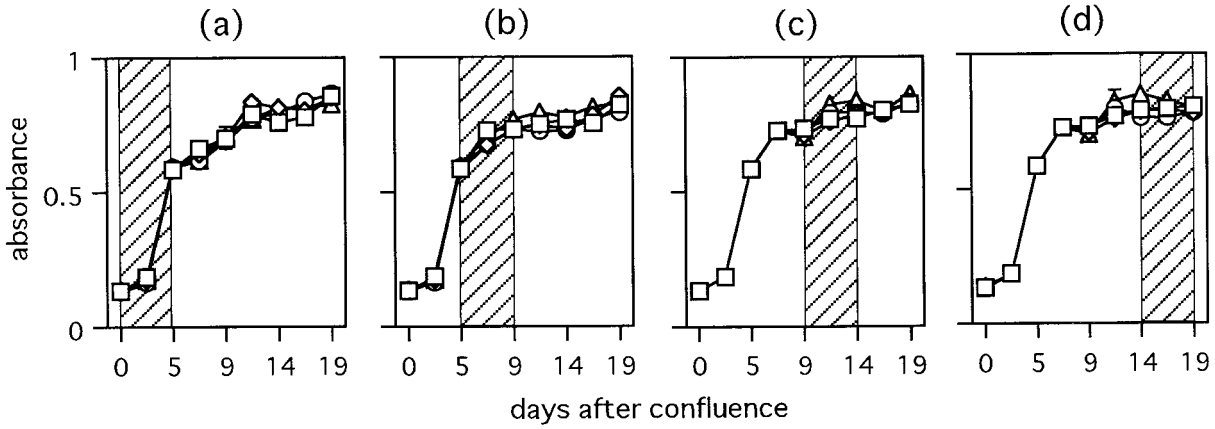

Fig. 1. Effects on cell proliferation resulting from the addition of activin-A were observed using an MTT assay. Cells were treated with 10 (◇), 50 (○), or 100 (△) ng/ml activin-A (shaded area) on days 0–5 **(a)**, days 5–9 **(b)**, days 9–14 **(c)**, or days 14–19 **(d)**. For control groups (□), cells were treated with just the vehicle. No effects on cell proliferation by activin-A were observed in any doses or at any time. Each value is the mean ±SEM of six samples.

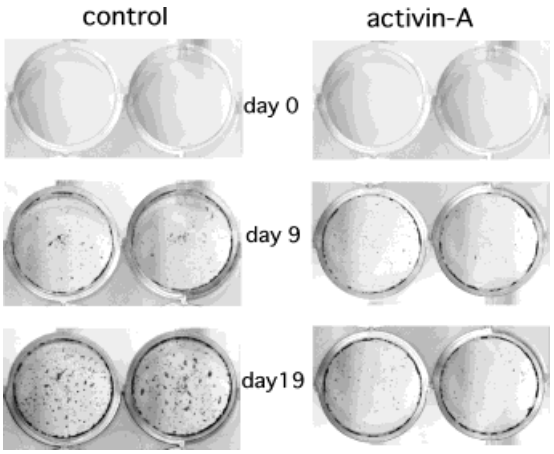

Fig. 2. Mineralized bone nodules after von Kossa staining on days 0, 9, and 19 in the control and on days 0–5 in the culture with 100 ng/ml of activin-A. Formation of bone nodules was inhibited by the addition of activin-A.

peaked on days 5 and 9 in the controls, respectively. Activin-A treatment suppressed the genes on days 5 and 9. OC mRNA levels gradually increased after confluence and peaked on day 14. Activin-A treatment resulted in a decrease in the expression on days 9 and 14 but resulted in an increase on day 19. The levels of actin mRNA was almost the same through this culture.

Both TGF-β1 mRNA and the βA-subunit (activin-A) mRNA were detected in the total cellular RNA from the FRC cells. TGF-β1 mRNA was expressed through the culture, and gradually increased (Fig. 6). On the contrary, the βA-subunit was limited to expression at an early stage of the culture (Fig. 7). Treatment of activin-A did not influence the levels of TGF-β1 mRNA expression.

DISCUSSION

The culture system of FRC cells was first reported by Bellows et al. [1986]. Since that report, several culture systems of osteoblastic FRC cells comprising cell differentiation, matrix formation, mineralization, and the expression of bone-related proteins have been reported [Antosz et al., 1989; Bellows et al., 1990a,b, 1995; Harris et al., 1994a,b; Malaval et al., 1995; Nefussi et al., 1997; Tang et al., 1996]. This culture system is a standard method for analyzing a series of proliferation and differentiation of the osteoblast-like cells. The content of the culture media after confluence is different from that before confluence, as described under Materials and Methods. In order to evaluate a series of effects of activin-A on the cultured cells in the same condition, we started to examine them including about proliferation just after confluence. In our experiments, cell proliferation continued from day 2 until day 9 or 11. The mineralized tissue, which is known as bone nodules, started to form on day 9, increasing thereafter. These results using our culture system were similar to those of previous reports. In addition, the time course of gene expression for bone-related proteins, including Col I, ALP, ON, OP, and OC, was similar to that noted in previous reports on FRC cells. The pattern of gene expression of these bone-related proteins is similar to that seen in bone formation in vivo, including sheep bone forma-

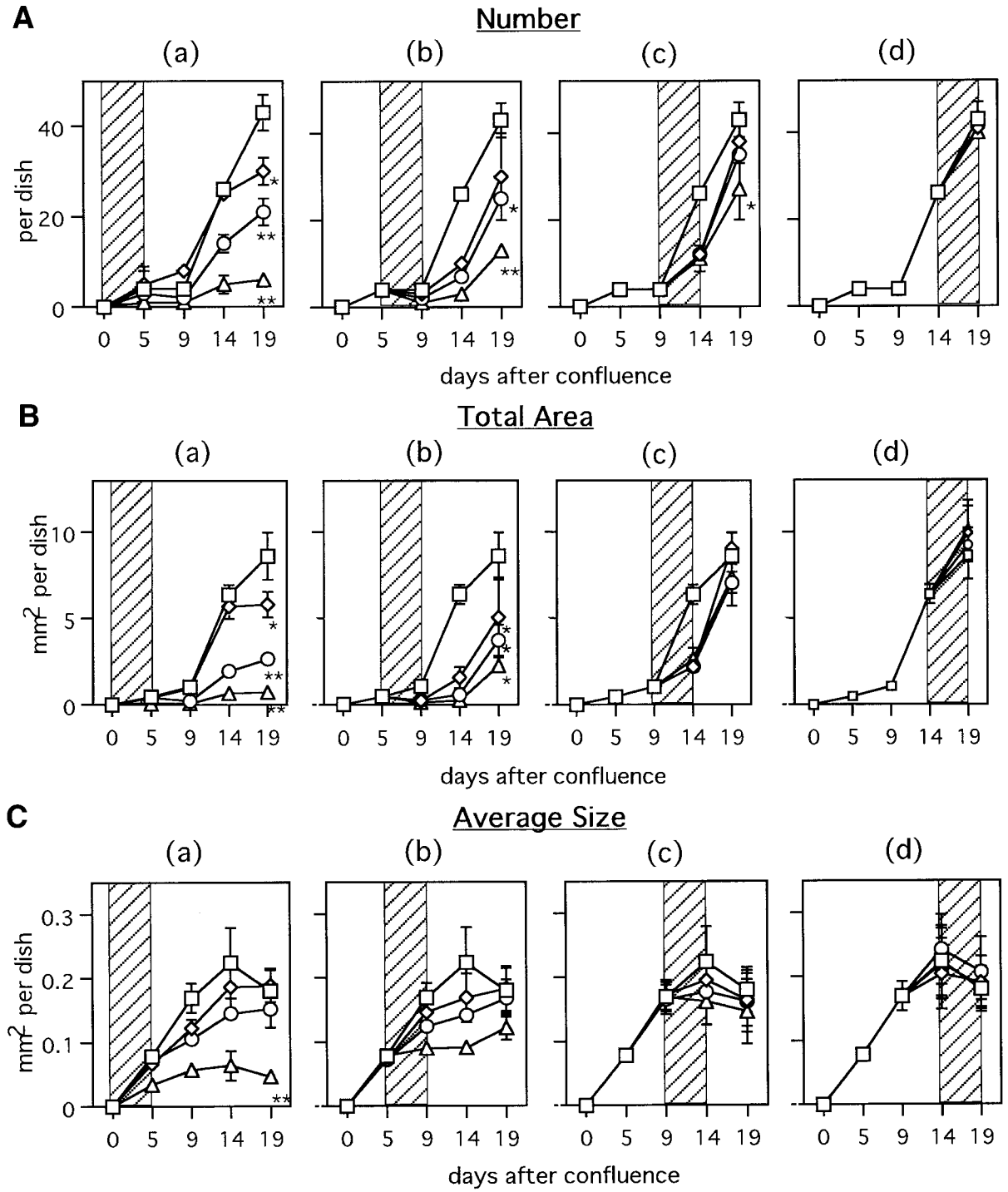

Fig. 3. Effects of activin-A on the numbers **(A)**, the total area **(B)** and the average size **(C)** of bone nodules in the culture of fetal rat calvarial (FRC) cells. 10 (◇), 50 (○), or 100 (△) ng/ml of activin-A (shaded area) were added on days 0–5 **(a)**, days 5–9 **(b)**, days 9–14 **(c)**, or days 14–19 **(d)**. For control groups (□), cells were treated with the vehicle alone. The addition of activin-A decreased the number, total area and average size of bone nodules in a dose-dependent manner, especially when activin-A treatment was on days 0–5. Activin-A did not influence the number, total area, and the average size of bone nodules when activin-A treatment was on days 14–19. Each value is the mean ±SEM of six samples. $*P < 0.05$ and $**P < 0.001$ compared with the control.

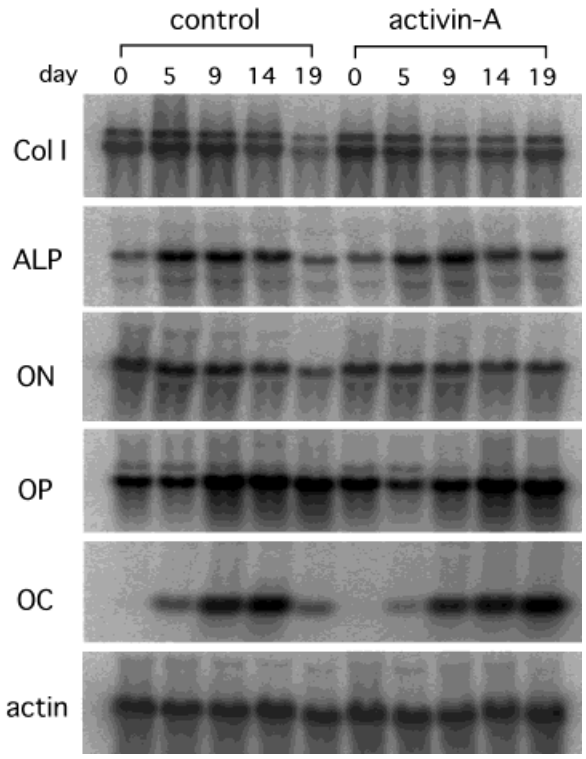

Fig. 4. mRNA levels for bone-related proteins of fetal rat calvarial (FRC) cells during culture treated with 100 ng/ml activin-A or with just vehicle (control) on days 0–5 in Northern blot analysis. Col I, type I (α1) procollagen (4.7 and 5.7 kb); ALP, alkaline phosphatase (2.6 kb); ON, osteonectin (2.2 kb); OP, osteopontin (1.5 kb); OC, osteocalcin (0.8 kb); actin (loading control; 2.1 kb).

tion [Zhou et al., 1994], rat fracture repair [Jingushi et al., 1992], and mouse embryogenesis [Ikeda et al., 1992], suggesting that this culture system using FRC cells accurately reflects bone formation in vivo.

Exogenous activin-A did not influence cell proliferation during the culture of FRC cells using the MTT assay. In addition, no effects were observed on cell proliferation despite changing the period of activin-A treatment. The MTT assay is nonradioactive and useful for analyzing cell proliferation during the long-term culture of FRC cells, as well as the ^{3}H thymidine incorporation assay. Using this MTT assay, only living cells are detected. Activin-A, like other growth factors of the TGF-β superfamily, is known to exert divergent effects on the proliferation and differentiation of various cells [Ueno et al., 1990]. In the MC3T3-E1 osteoblastic cell line, it is reported that the effects of activin-A on DNA synthesis depend on cell density, serum concentration, or the phase of differentiation [Hashimoto et al., 1992]. In addition, primary cultured osteoblasts from rat parietal bone are shown to have 8,000 high-affinity binding sites for activin-A [Centrella et al., 1991], and activin-A has mitogenic activity for those cells. Our results regarding the effects of activin-A on cell proliferation were different from such earlier reports. Our culture conditions were different from those used with the MC3T3-E1 osteoblastic cell line. The cell density was dense at the beginning of treatment with activin-A, and the serum concentration was relatively high. In the rat parietal bone cell culture in the previous reports, DNA synthesis is observed just before the confluent stage of the cultured cells. In our culture system, the effects of activin-A were investigated after confluence. This seems to be the reason activin-A did not influence cell proliferation after confluence during our long-term cultures of FRC cells.

Activin-A treatment impaired the number, total area, and average size of mineralized bone nodules in a dose-dependent manner. In addition, activin-A inhibited gene expression for matrix proteins including Col I, ON, OP, and ALP. Synthesis of these matrix proteins is a necessary step for bone nodule formation. A decrease in matrix protein synthesis appears to result in a decrease in bone nodule formation. This finding indicates that activin-A inhibited osteoblast differentiation. In addition, there were many nonmineralized nodules in the activin-A treated cultures (date not shown). It also demonstrates that activin-A did not influence cell proliferation after confluence and inhibited osteoblast differentiation.

TGF-β is previously reported to be a negative regulator of the FRC cell differentiation by inhibiting the expression of mRNA of bone morphogenetic protein 2 (BMP-2) [Harris et al., 1994]. Activin-A also inhibited the differentiation of FRC cells, similar to TGF-β, and it may inhibit the expression of BMP-2. Activin-A treatment did not influence the TGF-β mRNA of the FRC cells, indicating that this inhibitory effect was not due to any indirect actions by TGF-β.

The inhibitory effects of activin-A on bone nodule formation were significant when the cultured cells were treated by activin-A at the early phase of culture. These data suggest that activin-A inhibited early differentiation of FRC cells. In addition, type I activin receptor mRNA was detected during the early phase of the FRC cell culture using polymerase chain reaction

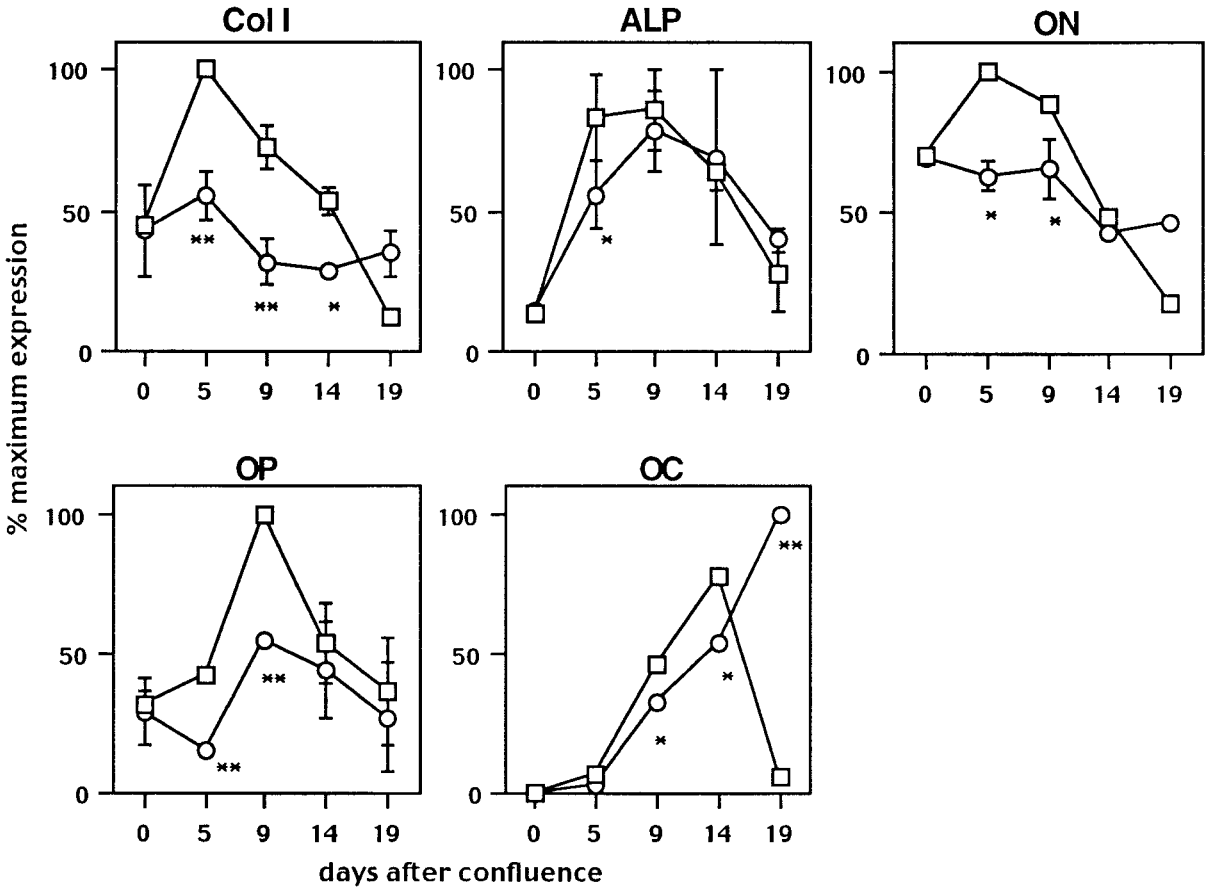

Fig. 5. Relative mRNA expression for bone-related proteins of fetal rat calvarial (FRC) cells during culture with 100 ng/ml activin-A (○) or with just the vehicle (control) on days 0–5 (□). Col I, type I (α1) procollagen; ALP, alkaline phosphatase; ON, osteonectin; OP, osteopontin; OC, osteocalcin. Activin-A treatment influenced mRNA expression for bone-related proteins. Each value is the mean ±SEM of three samples. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with the control.

(data not shown). Activin receptors are reported to be expressed in rat fracture calls during the early phase of intramembranous and endochondral bone formation [Shuto et al., 1997]. This indicates that exogenous activin-A directly acted on early cultured FRC cells or immature osteoblasts to inhibit further differentiation. In a previous report, accumulation of Col I is found to be a necessary component for osteoblast differentiation using FRC cell culture [Lynch et al., 1995]. Exogenous activin-A also impaired the expression of Col I in our FRC cell culture; this decrease in Col I may be another reason for the inhibition of further osteoblast differentiation.

Although activin-A inhibited the expression of bone related-proteins, Col I, ALP, ON, and OP, the expression of OC increased on day 19 compared with that seen in the controls. It was previously reported that OC is a marker of terminal differentiation for osteoblasts or is synthesized by mature osteoblasts. As described above, activin-A receptor was expressed during the early phase of the culture when the OC gene was not yet expressed. Therefore, it is difficult for activin-A to directly influence OC expression. Inhibition of early differentiation caused a delay in the differentiation process of the FRC cells by activin-A, resulting in a delay in the peak level of OC expression.

Activin-A is reported to be a stimulator of bone formation in vivo [Oue et al., 1994], whereas in the FRC cell culture system, activin-A inhibited bone nodule formation and mineralization. TGF-β shares certain similarities in that it is such a stimulator of bone formation in vivo [Noda and Camilliere, 1989] but inhibits osteoblast differentiation in vitro [Antosz et al., 1989; Harris et al., 1994]. Activin is thought to affect a different cell state in vivo than that present in this culture system like TGF-β.

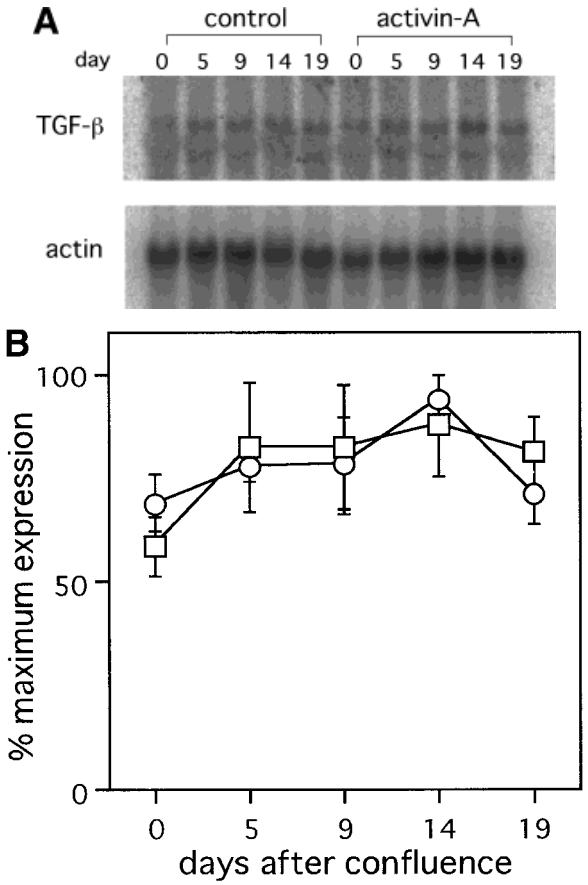

Fig. 6. **A:** mRNA levels for TGF-β1 in culture of fetal rat calvarial (FRC) cells during culture treated with 100 ng/ml activin-A or with the vehicle alone (control) on days 0–5. **B:** Relative mRNA expression for TGF-β1 of FRC cells during culture treated with 100 ng/ml activin-A (○) or with just the vehicle (control) (□) on days 0–5. Activin-A treatment did not influence mRNA expression for TGF-β1. Each value is the mean ±SEM of three samples.

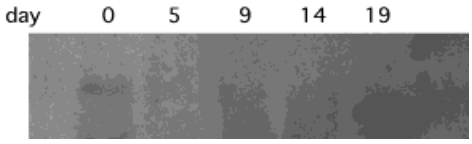

Fig. 7. mRNA levels for the βa-subunit in culture of fetal rat calvarial (FRC) cells during culture, not treated with activin-A (control). The βa-subunit mRNA was limited to expression at an early stage of culture.

Previous reports have shown that activin-A is in bone tissue and is expressed by osteoblasts [Ogawa et al., 1992]. The βA subunit mRNA, the homodimer of which is activin-A, was expressed during the early phase of the FRC cell culture. In addition, receptors for activin-A were expressed simultaneously by the FRC cells. These data indicate that activin-A has some roles to play in osteoblast proliferation or differentiation. In summary of our data, exogenous activin-A inhibited early differentiation of the FRC cells but did not influence cell proliferation. This suggests that the role of activin-A is to inhibit any further differentiation of premature osteoblasts, and activin-A may hold the premature state of the cells in the proliferating condition. Activin-A may suppress the initiation of differentiation and may support mitogenic activity by other cytokines, similar to TGF-β.

ACKNOWLEDGMENTS

We thank Dr. Y. Eto, Ajinomoto Central Research Laboratory, Ajinomoto Co., Inc., for kindly providing the human recombinant activin-A. The English used in this manuscript was revised by Miss K. Miller (Royal English Language Center, Fukuoka, Japan).

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