EXPERIMENTAL STUDY

Expression and localization of activin receptors during endochondral bone development

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Abstract

The expression and localization of activins (dimeric protein of inhibin β subunit) and activin receptors in skeletal tissue were examined. RT-PCR revealed that cultured chondrocytes expressed mRNAs of inhibin/activin β_A and four activin receptors (two type I (ActRI and ActRIB) and two type II (ActRII and ActRIIB)). Immunohistochemical analyses showed that activin β_A , ActRI and ActRII were localized in proliferating chondrocytes and osteoblasts in tibiae of neonatal rats, and in implants of demineralized bone matrix, a well-established model of ectopic bone formation. The immunoreactivities of osteoblasts were decreased with aging in the tibiae and with progressing endochondral bone development in the implants. The strong expression of ActRI was also detected in hypertrophic chondrocytes both in the tibial growth plate and in the implants, whereas immunoreactive ActRII was lower in hypertrophic chondrocytes. Western blot analysis also showed that immunoreactive ActRI, migrating at 52 kDa, was detected only in the implants on days 9 and 11, the period of conversion from cartilage to bone. In view of the sharing of type II receptors between activins and bone morphogenetic proteins (BMPs), our findings suggest that activin/BMP activity involves in bone modeling, especially during active chondro- and osteogenesis and during the conversion from cartilage to bone.

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Introduction

Activins, dimeric proteins of inhibin β subunits and members of the transforming growth factor- β (TGF- β) superfamily, were originally purified from follicular fluid as stimulators of the release of follicle-stimulating hormone from the pituitary (for review, see (1)). In common with other members of the TGF- β superfamily such as TGF- β s and bone morphogenetic proteins (BMPs), activin β subunit (β_A to β_E) mRNAs are widely distributed throughout the body, including the skeletal tissue (1), and activins are therefore presumed to act as local growth and differentiation factors in the bone. Activin A, a homodimer of inhibin β_A subunits, has indeed been isolated from bone matrix (2), and found to enhance both ectopic endochondral bone formation by BMPs and intramembranous bone formation in rats (2, 3). In addition, activin A induced chondrogenic differentiation in chick limb-bud cells (4) and enhanced the expression of type II collagen by chondrocytes *in vitro* (5). Furthermore, we have demonstrated that immunoreactive activin molecules were present in proliferating chondrocytes and round osteoblasts, and

that local injections of activin A stimulated cartilage formation, using an *in vivo* model of endochondral bone development induced by implantation of demineralized bone matrix (DBM) (6). All these findings suggest that activins, probably in concert with other systemic and local factors, have a significant role in cartilage and bone formation.

Activin signaling is initiated by the heteromeric cell-surface receptors, composed of type I and type II receptor subunits; four activin receptors, two type I receptors (ActRI and ActRIB) and two type II receptors (ActRII and ActRIIB), are known to form receptor complexes with activin (1, 7, 8). Each subunit is a transmembrane protein that is endowed with a cytoplasmic serine/threonine kinase domain. Activin binds directly to the extracellular domain of the type II receptor, followed by recruitment of the type I receptor. The type II receptor kinase then phosphorylates the type I receptor kinase in the cytoplasmic regulatory sequence, termed the GS domain; this phosphorylation activates the type I receptor kinase, which is responsible for the downstream signal transduction (1, 7, 8). Therefore, to understand the role and mechanism of

activins in the skeletal tissue, the identification and localization of activin receptors must first be established.

Shuto *et al.* (9) showed that immunoreactive ActRII was localized in osteoblasts at regions of intramembranous and endochondral bone formation. Taken together with our previous finding that active osteoblasts were activin β_A -positive (6), this suggests that activin acts as an autocrine factor. Unfortunately, however, Shuto *et al.* (9) were unable to examine the immunolocalization of the type I receptor of activin, the downstream transducer of the type II receptor for activin signaling, because of the unavailability of antibody against rat type I activin receptors. Furthermore, the distribution of type II activin receptors in the cartilaginous tissue was unknown (9). We have previously produced and characterized both anti-ActRI and anti-ActRII antibodies (10). In the present study, we examined the expression and localization of type I and type II receptors of activin in bone.

Materials and methods

Materials

Polyclonal antibodies directed against synthetic peptide of inhibin/activin β_A subunit (amino acids 87–99), ActRI (amino acids 81–89) or ActRII (amino acids 91–100) were kindly provided by Dr Kunio Torii, Ajinomoto Co., Kawasaki, Japan. The antibody to inhibin/activin β_A recognized activin A, but not inhibin A, on Western blot under non-reducing conditions (11), therefore we refer to this antibody as anti-activin β_A antibody. In addition, this antibody cross-reacted with neither TGF- β_1 , TGF- β_2 , nor rat albumin (11). The anti-ActRI antibody clearly recognized type I activin receptor after incubation with peptide N-glycosidase F (PNGase F) (10). Furthermore, these antibodies can be used for immunohistochemistry: gonads, brain and pancreatic islets, the tissues known to express activin and activin receptors, were stained positively by this method (6, 10, 12, 13), and the immunodistributions of activin β_A molecules in testis and ActRII molecules in brain were consistent with the expression of mRNA evaluated by *in situ* hybridization (10, 12, 14, 15).

Biotinylated anti-rabbit IgG antibody and avidin–biotin complex reagent were purchased from Vector Laboratories (Burlingame, CA, USA), and peroxidase-conjugated anti-rabbit IgG antibody was purchased from EY Laboratories (San Mateo, CA, USA). PNGase F from *Flavobacterium meningosepticum*, an enzyme that cleaves all types of asparagine-bound N-glycans (16), was purchased from Boehringer Mannheim (Indianapolis, IN, USA). DBM of particle size 74–420 μm was prepared from diaphyses of femur and tibia of adult rats as described by Reddi & Huggins (17).

Chondrocyte culture

Proximal tibiae from 4-day-old Wistar rats (Japan Laboratory Animals, Tokyo, Japan) were rinsed in Ca^{2+} , Mg^{2+} -free phosphate-buffered saline and digested with 0.1% trypsin for 15 min at 37 °C, followed by 0.2% collagenase and 0.2% trypsin for 4 h at 37 °C. Dissociation of cells was facilitated by repeated pipetting. Chondrocyte-rich cells were harvested by low-speed centrifugation (1000 r.p.m. for 5 min) and resuspended in DME/F12 culture medium containing 10% fetal bovine serum and 6 mg/l kanamycin supplemented with 50 $\mu\text{g}/\text{ml}$ L-ascorbic acid (18). The 1.0×10^6 cells were plated in 9-cm plastic dishes. The chondrocyte-enriched culture (>90%) was checked by cell shape and toluidine blue staining (data not shown). During the logarithmic growth phase (at about 70% confluence), cells were harvested and mRNA was extracted for examination of the expression of activin receptors by RT-PCR analysis.

Animals and developing endochondral bone

For examination of the immunolocalization of activin β_A and activin receptor molecules, tibiae of rats aged 0, 4, 7, 10 and 14 days, 5 weeks and 3 months were collected after the animal was killed by decapitation. Tibiae from at least three rats at each time point were subjected to immunohistochemistry.

To examine changes in the expression of activin receptors during bone development, we used male Sprague–Dawley rats (Charles River, Atsugi, Japan) weighing 100–110 g. They were cared for according to the principles outlined in the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (19). Endochondral bone development was induced in rats by s.c. implantation of 30 mg DBM on each side of the thoracic region. The implantation was conducted under light diethyl ether inhalation anesthesia. The implants were dissected out 5, 7, 9, 11, 14 and 21 days after implantation. For immunohistochemistry, at least six implants were immersed in Bouin's solution at each time point. For Western blot analysis, six to eight implants were pooled, because a large amount of DBM was required for detection of immunoreactive activin receptor molecules.

RT-PCR

Poly(A)-RNA extraction and synthesis of cDNA were conducted using commercial kits (QuickPrep Micro mRNA Purification kit, Pharmacia Biotech, Uppsala, Sweden; SuperScript Preamplification System, Life Technologies, Gaithersburg, MD, USA). RT-PCR analysis was conducted to identify mRNAs of inhibin/activin β_A subunit and four activin receptors in cultured chondrocytes. Table 1 shows oligonucleotide primers and the predicted sizes of the PCR products. The

Table 1 List of primers for detection of inhibin β_A and active receptor mRNAs.

Gene of interest (fragment size)	Forward primer	Reverse primer
Inhibin/activin β_A (165 bp)	rhnh β_A 1264(+): 5'-TCAACAGTCATTAAACCACCTACCGCATGA-3'	rhnh β_A 1429(-): 5'-AGCCACACTCCTCCACAATCATGTT-3'
ActRI (545 bp)	rActRI 875(+): 5'-ATGACTACCTTCAGCTCACC-3'	rActRI 1419(-): 5'-CTTCGCCAGAGAAGTTAATG-3'
ActRII (629 bp)	rActRII 884(+): 5'-ACCGCTACACAGTGACCAT-3'	rActRII 1512(-): 5'-CTTCAGCTCTTCTGCACGCT-3'
ActRIII (703 bp)	rActRIII 835(+): 5'-CTTAAGGCTAATGTGGTCTC-3'	rActRIII 1537(-): 5'-GACTAGATTCTTTGGGAGGA-3'
ActRIIB (857 bp)	rActRIIB 137(+): 5'-ACCGACATCGAAAGCCTCCC-3'	rActRIIB 993(-): 5'-CAGCCAGTGATCCTTAATG-3'

reaction mixtures (50 μ l; cDNA 37.5 ng, primers 20 pmol each, dNTP 10 nmol, Ex-Taq DNA polymerase (Takara, Otsu, Japan) 1 U) were pre-cooled on ice for 10 min (cool start PCR), then cycled 32 times with a cycle profile of 60 s at 94 °C, 60 s at 57 °C, and 60 s at 72 °C, followed by a 10-min extension at 72 °C using a Perkin-Elmer DNA thermal cycler (Norwalk, CT, USA). The products were separated on a 3% agarose gel and visualized with ethidium bromide, and bands corresponding to the expected sizes were recovered from the gel with SUPREC-01 (Takara, Otsu, Japan). Recovered fragments were cloned into a pCR2.1 vector (Invitrogen, San Diego, CA, USA), and sequenced using Dye Terminator Cycle Sequencing Ready Reaction kit (Foster City, CA). To avoid false-positive results from contamination by genomic DNA, samples with or without treatment of ribonuclease-H-free reverse transcriptase were prepared simultaneously. As a further negative control, RT-PCR without RNA samples was also carried out.

Immunohistochemistry

After fixation with Bouin's solution for 4 h at room temperature, the implants were dehydrated, embedded in paraffin, and sectioned at 5- μ m thickness. The tibiae and the implants at 21 days after implantation were embedded after decalcification with 10% EDTA (pH 7.4) for 3–10 days at 4 °C. The step of decalcification did not affect the immunoreactivity, which was verified in the tibiae of newborn rats using anti-activin β_A antibody (data not shown). The avidin–biotin–peroxidase procedure was performed as previously (6, 12). After endogenous peroxidase had been blocked with 0.3% H₂O₂ in methanol for 30 min, the sections were treated with 1 mg/ml hyaluronidase, buffered to pH 5.5 with 0.1 mol/l sodium acetate in 0.15 mol/l NaCl for 30 min. Binding of antibody, diluted 1:200 for anti-activin β_A antibody or 1:100 for anti-ActRI or ActRII antibody, was visualized using biotinylated goat anti-rabbit IgG (1:100) for 30 min, followed by avidin–biotin complex reagent for 45 min and development with 0.05% 3,3'-diaminobenzidine tetrahydrochloride for 5–10 min. To confirm the method, sections without primary antibody or secondary antibody were stained as negative controls. In addition, non-immune normal rabbit serum or primary antiserum preadsorbed with excess corresponding peptides was used as negative control.

Protein preparation and Western blot

The homogenization of implants and protein preparation after extraction into a solution containing 4 mol/l guanidine hydrochloride were performed as described previously (6). The anti-ActRI antibody could detect a 52 kDa band only when protein was preincubated with PNGase F (10). For cleavage of carbohydrate chains

from proteins, 50 µg of the proteins were incubated with or without 0.5 U PNGase F in 6 µl 42 mmol/l phosphate buffer and 10 mmol/l EDTA (pH 7.4) for 16 h at 37 °C. The digested products were analyzed by Western blot.

Fifty micrograms of the proteins were run on 10% polyacrylamide SDS-PAGE, and Western blot analyses were conducted as described previously (10). Almost equal among-lanes efficiencies in transfer of proteins to the membrane were checked by Coomassie brilliant blue staining of gels after electroblotting. Anti-ActRI or anti-ActRII antibody at a 1:1000 dilution was reacted with proteins transferred to the membrane for 16 h at 4 °C. After incubation with secondary antibody (peroxidase-conjugated anti-rabbit IgG antibody), the reacted proteins were visualized using enhanced chemiluminescence reagent (ECL detection kit, Amersham, Aylesbury, Bucks, UK). Western blot analyses were repeated three times using each antibody. Specificity of the bands was examined using non-immune normal rabbit serum and using the antibodies preadsorbed with excess corresponding peptides.

Results

Detection of inhibin/activin β_A subunit and type I and type II activin receptor mRNAs in cultured chondrocytes and osteoblasts

Not only inhibin/activin β_A subunit mRNA but also four activin receptor mRNAs were detected in cultured chondrocytes by RT-PCR (Fig. 1). PCR products with the expected size were obtained in samples treated with reverse transcriptase. In the absence of reverse transcriptase or RNA samples during the synthesis of cDNA, no bands were detected (data not shown). The subcloning and sequencing confirmed them to be the expected cDNAs of inhibin/activin β_A subunit and activin receptors.

Immunolocalization of activins and activin receptors in the skeletal tissue

RT-PCR analysis suggested that cultured chondrocytes expressed mRNAs of inhibin/activin β_A and both types

Figure 1 Detection of rat inhibin/activin β_A and activin receptor mRNAs in cultured chondrocytes. Primary cultures were made of chondrocytes from proximal tibiae. During the logarithmic growth phase, cells were harvested and mRNAs were extracted and reverse transcribed. PCR was performed using primers for inhibin/activin β_A (β_A), ActRI (I), ActRII (IB), ActRII (II) or ActRII (IIB), with 32 cycles of 60 s at 94 °C, 60 s at 57 °C, and 60 s at 72 °C. The products were separated on 3% agarose gel and visualized with ethidium bromide.

of activin receptor. We next examined immunolocalization and age-related changes in expression of activin β_A , ActRI and ActRII in the skeletal tissue. Although we have also previously produced three kinds of anti-ActRIIB antibody, characterization by Western and ligand blot analyses revealed that these antibodies did not react significantly with proteins of the expected size, or recognized not only activin receptor but also non-specific proteins of 53 kDa and 56 kDa that could not bind with activin A (10). Therefore, we did not attempt to localize ActRIIB. In addition, useful anti-ActRIIB antibodies were not available.

Changes in the histochemical localization of immunoreactive activin β_A , ActRI or ActRII with age in the proximal tibiae are shown in Table 2. When non-immune control serum was used instead of primary antibody, no reaction was found in the sections (data not shown). As shown in Fig. 2A–C, immunoreactive

Table 2 Changes in immunoreactive activin β_A and activin receptors in proximal tibiae with growth in rats.

Antibody:	Age																				
	0 days			4 days			7 days			10 days			14 days			5 weeks			3 months		
	β _A	I	II	β _A	I	II	β _A	I	II	β _A	I	II	β _A	I	II	β _A	I	II	β _A	I	II
Resting chondrocyte	±	+	+	+	+	+	+	++	±	±	++	+	±	++	±	+	++	+	±	—	±
Proliferating chondrocyte	++	++	+	++	++	+	++	++	+	+	++	+	+	++	+	++	++	±	++	+	±
Hypertrophic chondrocyte	—	—	—	+	—	—	+	+	±	±	+	±	—	++	±	±	++	±	—	±	—
Osteoblast	++	++	++	+	++	++	+	±	±	+	±	±	+	±	±	—	—	—	±	±	±

β_A , Anti-activin β_A antibody; I, anti-ActRI antibody; II, anti-ActRII antibody. The intensity of reaction is given on the scale: –, negative; ±, only slightly positive; +, moderately positive; ++, intensely positive.

Figure 2 Immunolocalization of activin β_A and activin receptors in tibiae of growing rats. The tibiae were fixed in Bouin's solution and decalcified with 10% EDTA solution. Paraffin-embedded sections were stained with anti-activin β_A antibody (A–C), anti-ActRI antibody (D–F) or anti-ActRII antibody (G–I), using biotinylated anti-rabbit IgG antibody, avidin-biotin complex reagent, and 3,3'-diaminobenzidine tetrahydrochloride. Original magnification, $\times 75$. High magnification view ($\times 300$) of the boxed area of (A) and (E) are presented in (J) and (K), respectively. When non-immune serum was used instead of primary antibody, no reaction was found in the sections. Positive reactions were seen in proliferating chondrocytes in the tibiae of newborn rats (A, D and G), those of rats aged 14 days (B, E and H) and those of rats aged 5 weeks (C, F and I). Cells resembling osteoblasts in tibial metaphyses of newborn rats were also intensely stained (A, D, G and J). Note the intense reactions to anti-ActRI antibody in hypertrophic chondrocytes of the tibial growth plate (E, F and K). Pz, proliferating zone of growth plate; Hz, maturing and hypertrophic zone of growth plate; B, bone; *, proliferating chondrocytes; arrowhead, hypertrophic chondrocytes; arrows, cells resembling osteoblasts.

Figure 3 Immunolocalization of activin receptors in the implants. Thirty milligrams DBM were implanted s.c. into the thoracic region of growing rats. The implants were fixed in Bouin's solution. Paraffin-embedded sections were stained with anti-ActRI antibody (A and B) or anti-ActRII antibody (C and D), using biotinylated anti-rabbit IgG antibody, avidin-biotin complex reagent, and 3,3'-diaminobenzidine tetrahydrochloride. Positive reactions were seen in chondrocytes on day 7 of implantation (A and C) and in osteoblasts on day 9 of implantation (B and D). Furthermore, hypertrophic chondrocytes were intensely stained by anti-ActRI antibody (B) but not by anti-ActRII antibody (D). When non-immune serum was used instead of primary antibody, no reaction was found in the sections (E and F). Furthermore, when the antiserum was absorbed with an excess amount of corresponding peptides, the reaction was abolished. Original magnification, $\times 330$. M, implanted bone matrix; * proliferating chondrocytes; arrowheads, hypertrophic chondrocytes; arrows, osteoblasts.

activin β_A molecules were detected in the growth plate and metaphyses. In newborn rats, proliferating chondrocytes in the proximal tibia, but not hypertrophic chondrocytes, were stained (Fig. 2A). In addition, cells resembling osteoblasts in the metaphyses were also stained (arrow in Fig. 2J), although other types of cell were also stained. Similar distribution of immunoreactive activin β_A was also detected in the proximal tibia in rats aged 4 days, although the immunoreactivity in osteoblasts was decreased (data not shown). In rats aged 14 days, proliferating chondrocytes still reacted with anti-activin β_A antibody, but the reactivity tended to decrease (Fig. 2B). In rats aged 5 weeks, the immunoreactivity of osteoblasts had disappeared (Fig. 2C), although bone matrix was strongly stained.

The immunolocalization of activin receptors in skeletal tissue is presented in Fig. 2D–I. The distribution of ActRI-positive cells was similar to that of activin β_A and ActRII, but included some distinct regions. In newborn rats, proliferating chondrocytes in the growth

plate and osteoblasts in the metaphyses were strongly stained by anti-ActRI antibody (Fig. 2D), and positive reaction in osteoblasts disappeared in the bone of rats aged 5 weeks (Fig. 2F); this immunodistribution was similar to those of activin β_A and ActRII. The most remarkable feature of immunostaining of ActRI was that the molecules were strongly detected in hypertrophic chondrocytes in rats aged 14 days (Fig. 2E and arrowhead in Fig. 2K) to 5 weeks (Fig. 2F), although hypertrophic chondrocytes in the tibiae of newborn rats were ActRI-negative (Fig. 2D). These reactions were abolished by preadsorption of antibody with excess of the corresponding peptides (data not shown). This distinctive reaction could not have been caused by the difference of a greater concentration of antibody, in view of the comparable background and negative reaction in newborn rats.

The distribution of ActRII-positive cells was essentially similar to that of activin β_A -positive cells – that is, the positive cells were proliferating chondrocytes and

Figure 4 Time course of changes in immunoreactive activin receptors during endochondral bone development. The implants were obtained as described in Fig. 3. Fifty micrograms of proteins were electrophoresed and transferred to the polyvinylidene difluoride membrane. The membranes were incubated with anti-ActRI antibody (A) or anti-ActRII antibody (B). The details of Western blot analysis are shown in Materials and Methods. The arrow indicates 52 kDa.

osteoblasts in tibiae of newborn rats (Fig. 2G). These reactions were eliminated by use of the antibody preadsorbed with excess of the corresponding peptides (data not shown). With aging, the positive reaction in osteoblasts weakened and disappeared in rats aged 5 weeks (Fig. 2I), although hypertrophic chondrocytes were only slightly stained in tibiae of rats aged 7 days (data not shown) to 5 weeks (Fig. 2I). In the resting chondrocytes, immunoreactive activin β_A , ActRI and ActRII molecules were mainly localized in the nucleus.

Expression of activin receptors during endochondral bone development

To examine changes in the expression of activin receptors during endochondral bone development more extensively, we used a model of ectopic bone formation induced by implantation of DBM in rats (6, 17). In this model, prechondrocytes were seen on day 5, and chondrocytes were abundant on day 7 of implantation. On day 9, hypertrophic chondrocytes and several chondroclasts in the vicinity of degenerating chondrocytes appeared, and osteoblasts were evident on the surface of calcified cartilage spicules and the implanted matrix particles. On day 11 of implantation, cartilaginous tissue was smaller and bone-like tissue was larger. Active bone remodeling by osteoblasts and osteoclasts was evident on day 14, and bone marrow was formed on day 21 (6, 20). All these histological changes are

indistinguishable from those seen in most bone development and in fracture healing (20).

Similar to their immunodistribution in bone, both anti-ActRI and ActRII antibodies stained strongly in proliferating chondrocytes in implants on day 7 (asterisk in Fig. 3A and C). In addition, on day 9, all types of cells, including hypertrophic chondrocytes and round osteoblasts, were strongly stained by anti-ActRI antibody (arrowhead and arrow in Fig. 3B). In contrast, hypertrophic chondrocytes were weakly stained by this antibody (arrowhead in Fig. 3D), although round osteoblasts were strongly stained by anti-ActRII antibody (arrow in Fig. 3D). No significant reaction was detected by use of non-immune serum instead of primary antibody (Fig. 3E and F). Furthermore, when the antibody was absorbed with an excess amount of corresponding peptide, the reaction was abolished (data not shown). In implants on day 21, no cells showed positive reaction to anti-ActRI or ActRII antibody (data not shown).

We also examined expression of activin receptors during ectopic bone development, using Western blot analyses. When anti-ActRI antibody was used, a significant band at 52 kDa was detected only on days 9 and 11 (Fig. 4A). Furthermore, bands at 60 kDa were also seen in implants from days 5 to 11, and the band intensity of implants on days 9 and 11 was stronger. All these bands were eliminated by preadsorption of antibody with the excess corresponding peptides. In view of the molecular size, the band at 52 kDa would indicate ActRI (10). In addition to these bands, another band at 47 kDa was also seen, but this band was interpreted as being due to non-specific reaction because it was not eliminated by preadsorption of the antibody (Fig. 4A, lanes 7 and 8).

When anti-ActRII antibody was used, a broad band was seen at 58–62 kDa in implants on days 5–11; this is similar to the molecular size of ovarian ActRII (10). It was eliminated by preincubation of antibody with corresponding peptides (Fig. 4B). The bands were at least triple, and the band intensities at 58–62 kDa were comparable among implants on days 5–11. Although it was not clear, the band at 60 kDa for ActRI and triplet bands for ActRII may result from incomplete deglycosylation of activin receptors and subsequent different migration on SDS-PAGE.

Discussion

The present study has demonstrated the expression of activin and its receptors in chondrocytes and osteoblasts. Cultured chondrocytes expressed not only activin β_A mRNA but also mRNAs of four activin receptors (both type I (ActRI and ActRIB) and type II (ActRII and ActRIIB)). In addition, analyses using anti-ActRI and anti-ActRII antibodies revealed that activin receptors were localized in proliferating chondrocytes

and osteoblasts in developing bone and in the implants of DBM. In view of the binding of BMPs to ActRII and ActRIIB and signaling via ActRI in response to BMPs (21–23), our findings suggested that these cells would be targets of activin/BMP.

Our results suggested that the activity of activin in osteoblasts may be higher during the rapid growing period of the bone. The immunoreactivity in osteoblasts was decreased within a week after birth. Nagamine *et al.* (24) showed that activin expression was greater near the osteoblasts on the surface of the newly formed trabecular bone during fracture healing. They also revealed that ActRI and ActRII were co-expressed in intramembranous and endochondral ossification sites (24). In addition, Dewulf *et al.* (25) and Ishidou *et al.* (26) demonstrated greater expression of type I receptors for BMPs in chondrocytes and osteoblasts during rapid bone formation. Furthermore, recent studies on localization of Smads, downstream mediators of the TGF- β family members (1, 27), revealed that Smad2 and Smad3 (activin/TGF- β -regulated Smads) were highly expressed in proliferating chondrocytes and maturing chondrocytes, respectively, and Smad1 and Smad5 (BMP-regulated Smads) were present in proliferating chondrocytes (28). In view of the stimulatory effects of activin on chondro- and osteogenic activities (2–6), all these findings suggested that activin would have a morphogenic role during endochondral bone formation in concert with the other TGF- β family members. Furthermore, a recent study revealed the synergistic effect of activin on receptor activator of NF κ B ligand-induced osteoclast formation (29). Activin may regulate bone turnover to maintain bone morphogenesis.

The most distinctive feature concerning the localization of activin receptors was that ActRI was intensely localized in hypertrophic chondrocytes in tibial growth plate of growing rats, although activin β_A and ActRII weakly localized there. This finding was consistent with those of a clinical study by Yonemura *et al.* (30), that hypertrophic chondrocytes in pathological ectopic ossified tissue of the paravertebral ligament were ActRI-positive. In the present study, the positive reaction was first detected in hypertrophic chondrocytes in the tibiae of rats aged 7 days and the reactions were strongest in rats aged 14 days to 5 weeks. The results of implants of DBM were also consistent with immunolocalization of ActRI in the growth plate. Hypertrophic chondrocytes, in addition to proliferating chondrocytes and emerging osteoblasts, were intensely stained by anti-ActRI antibody in implants on days 9 and 11. Furthermore, Western blot analyses showed that a band of 52 kDa, the size detected in ovarian protein in our previous study (10), was seen in implants only on days 9 and 11, the time corresponding to the period of existence of hypertrophic chondrocytes. In contrast to changes observed in immunoreactive ActRI molecules during endochondral

bone development, immunoreactive ActRII revealed by Western blot analyses was relatively constant in implants on days 5–11. In addition, no bands were detected on days 14 and 21, which was similar to the timing of ActRI expression. The different patterns of expression and localization between ActRI and ActRII suggest that diverse activities of activin/BMP may be elicited by different combinations of type I and type II receptors in proliferating and hypertrophic chondrocytes and osteoblasts.

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