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Regulation of Osteogenesis by Fetuin*

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Osteoporosis is a common problem of aging and results from a failure of homeostatic mechanisms to regulate osteogenesis and mineralization. Bovine and human forms of fetuin glycoprotein bind to the transforming growth factor (TGF)-β/BMP (bone morphogenic protein) cytokines and block their osteogenic activity in cell culture assays (Demetriou, M., Binkert, C., Sukhu, B., Tenenbaum, H. C., and Dennis, J. W. (1996) *J. Biol. Chem.* 271, 12755–12761). Fetuin is a prominent serum glycoprotein and a major noncollagenous component of mineralized bone in mammals. In this study, we show that recombinant fetuin and native serum protein have similar potency as inhibitors of osteogenesis in dexamethasone-treated rat bone marrow cell cultures (dex-RBMC). Recombinant bovine fetuin also bound to TGF-β1 and BMP-2 *in vitro* with kinetics similar to native fetuin. Although TGF-β1 is required for osteogenesis in dex-RBMC, the cytokine also inhibited osteogenesis at concentrations ≥10 pM. Titration of fetuin or anti-TGF-β1 antibodies into the bone marrow cultures in the presence of 10 pM TGF-β1 restored osteogenesis, whereas titrations of the same reagents into cultures with 0.3 pM added TGF-β1 were inhibitory, confirming the biphasic nature of the TGF-β1 response. Suppression of osteogenesis by both TGF-β1 and the antagonist proteins required their presence within the first 6 days of culture, well before mineralization at 10–12 days. Northern analysis showed that both fetuin and high dose TGF-β1 suppressed expression of the bone-associated transcripts alkaline phosphatase, osteopontin, collagen type I, and bone sialoprotein. The suppression of osteogenesis by fetuin and by high dose TGF-β1 was accompanied by the differentiation of an alternate cell lineage with adipocyte characteristics. In summary, the biphasic osteogenic response to TGF-β1 suggests that overlapping gradients of TGF-β/BMP cytokines and fetuin regulate osteogenesis in remodeling bone.

Bovine fetuin and the human homolog α2-HS-glycoprotein (AHSG)[1] are secreted by hepatocytes and constitute a major component of serum and the noncollagenous protein fraction of mineralized bone (1–3). Serum fetuin measured in cattle, sheep, and goats is highest in the 3rd trimester (*i.e.* 10–22 g/liter) and declines after birth by an order of magnitude in adults (reviewed in Ref. 4). Fetuin is a negative acute phase reactant (5), and serum levels decline in some cancer patients correlating with impaired cellular immune function (6).

The abundance of fetuin in bone suggests that the glycoprotein may have a role in bone formation or remodeling. Patients with Paget's disease, an affliction of increased bone turnover with disordered and thickened bone, show depressed serum AHSG levels (7). Elevation of serum AHSG has been correlated with a common form of mild osteogenesis imperfecta where bone mass is lost (8). Homozygosity for AHSG*1, a polymorphism in the *AHSG* gene (9), is associated with shorter stature and reduced bone quality as measured by calcaneal broadband ultrasound (10). Fetuin does not appear to be required for embryogenesis, as mice lacking the protein are viable with no gross anatomical abnormality at birth (11). However, fetuin-deficient mice show altered cellular and biomechanical properties in bone at 3 months of age.[2]

We have reported that fetuin binds to BMP-2 > BMP-4 > BMP-6 > TGF-β1 > TGF-β2 with K_D values ranging from 2×10^{-8} M to 2×10^{-6} M, respectively (12). Fetuin and the TGF-β receptor type II (TβRII) share sequence homology within a 19–20-amino acid disulfide-looped sequence designated TGF-β receptor homology domain (TRH1), which defines the major cytokine binding domain. Cyclized TRH1 peptides bind directly to TGF-β1 and BMP-2 cytokines and competitively inhibit TGF-β binding to TβRII. Fetuin blocked TGF-β1 activity in cell cultures with an IC_{50} of 1–2 μM in cell culture, similar to the K_D value measured by surface plasmon resonance. Fetuin concentrations in human serum are similar to the TGF-β1 binding constant, consistent with a role for fetuin in regulating TGF-β activity *in vivo* (12). Considering the higher affinity of fetuin for BMP cytokines than TGF-βs, the antagonist may also regulate their activities. The BMPs are important in skeletal development during embryogenesis (13) and induce ectopic bone formation when injected into animals (14).

TGF-β1 is concentrated in mineralized human bone but declines in concentration with age, and levels in bone matrix correlate with bone turnover rates (15). The release and activation of TGF-β1 by osteoclasts in bone matrix has been implicated in several sequential steps in bone remodeling: recruitment of osteoblast precursor cells, induction of matrix deposition, and mineralization. In mice, an age-related decline

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[1] The abbreviations used are: AHSG, α2-HS-glycoprotein; TGF-β, transforming growth factor-β; dex-RBMC, dexamethasone-treated rat bone marrow cells; AP, alkaline phosphatase, Coll I, collagen type I; BSP, bone sialoprotein; OPN, osteopontin; MBP, maltose-binding protein; MOPS, 4-morpholinepropanesulfonic acid; BMP, bone morphogenic protein.

[2] M. Szweras, B. Sukhu, M. Kasra, H. C. Tenenbaum, W. Jahnen-Dechent, M. D. Grynpas, and J. W. Dennis, manuscript in preparation.

in bone TGF-β1 is associated with reduced numbers and responsiveness of the bone marrow osteoprogenitor cells (16). A polymorphism in the coding region of the TGF-β1 gene has been correlated with decreased serum levels of TGF-β1 and susceptibility to osteoporosis in postmenopausal Japanese women (17). In cell culture models of osteogenesis, TGF-β1 appears to regulate sequential events including stimuli for osteoblast precursor cell recruitment and differentiation, cessation of cell proliferation, synthesis, and deposition of matrix proteins and mineralization (18). The addition of glucocorticoid (*i.e.* dexamethasone) to rat bone marrow cell cultures (dex-RBMC) enhances the activation of latent TGF-β, which correlates with osteogenic differentiation and expression of the osteoblast markers alkaline phosphatase (AP), osteopontin (OPN), bone sialoprotein (BSP) and collagen type I, as well as promoting mineralization (19).

In this report, we show that recombinant bovine fetuin, made as a fusion protein with maltose-binding protein (MBP-fetuin) in *Escherichia coli* has similar affinities for TGF-β1 and BMP-2 as the native protein as well as similar potency in regulating osteogenesis in dex-RBMC. Furthermore, we demonstrate that TGF-β1 produced in the cultures is necessary for differentiation, but that excess exogenous TGF-β1 completely inhibited mineralization. This biphasic response to TGF-β1 showed a pronounced optimum, and addition of both exogenous fetuin and neutralizing anti-TGF-β1 antibodies shifted the TGF-β1 optimum for osteogenesis. Both fetuin and high dose TGF-β1 inhibited dex-induced increases in AP, BSP, OPN, and Coll I transcripts, as well as mineralization while promoting the differentiation of cells with adipocyte characteristics. This suggests that suboptimal levels of TGF-β1 block osteogenic differentiation and are permissive for adipogenesis in dex-RBMC. Our results show that fetuin antagonizes TGF-β1 activity in dex-RBMC and suggests that gradients of cytokine and antagonists may regulate osteogenic and adipogenic cell differentiation in bone.

EXPERIMENTAL PROCEDURES

Materials—Bovine fetuin was purchased from Sigma (catalog #F3004), and bovine serum albumin was from Roche Molecular Biochemicals (fraction V). R&D Systems Research provided recombinant human TGF-β1 (catalog #240B), recombinant human sTβRII/Fc (catalog #341-BR), and anti-TGF-β1-neutralizing antibodies (catalog #AB-101-NA). Recombinant human BMP-2 was kindly supplied by Genetics Institute.

Fetuin Expression Construct—Bovine fetuin was expressed as an MBP fusion protein in the pMal-c2 vector (New England Biolabs). Full-length fetuin cDNA was cloned by polymerase chain reaction from a bovine liver cDNA library (Stratagene). Polymerase chain reaction-directed mutagenesis was used to connect the open reading frames of MBP and fetuin. The signal peptide of fetuin, which is the C-terminal fusion partner, was removed during this process. The amino acid sequence at the site of the fusion was IEGRISEFGSSRVENSIPLD. IEGR is the recognition sequence for factor Xa protease, which is present at the C terminus of MBP. IPLD represents the N terminus of native fetuin. The nucleotide sequence of the entire cDNA inserted into the expression vector was verified.

Expression, Extraction, and Purification of Protein—The MBP fusion protein was expressed in the *E. coli* strain AD494 (Novagen). An overnight culture of the cells carrying the MBP-fetuin expression construct was diluted 1/100 into 660 ml of LB medium (Difco, catalog #0446-17-3) containing 100 μg/ml ampicillin. The culture was grown at 37 °C to an A_{600} of approximately 0.9, at which point isopropyl-1-thio-β-D-galactopyranoside was added to the medium for a final concentration of 0.5 mM. Recombinant protein expression was induced for 2 h at 37 °C. The cells were harvested in a GSA rotor (4000 rpm for 20 min at 4 °C), resuspended in 17 ml of ice-cold lysis buffer (10 mM Na_2HPO_4 (pH 7.0), 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 10 mM EGTA, 10 mM β-mercaptoethanol), frozen in a 50-ml falcon tube in dry ice/ethanol, and stored at −20 °C. The extract was thawed on ice and sonicated on ice with an 8-mm tip for a total of 90 s in 2 intervals. 0.1 volume of 5 M NaCl was added, and cellular debris was removed by centrifugation (SS34 rotor, 12,000 rpm for 15 min at 4 °C). The supernatant was diluted into 5 volumes of column buffer (10 mM Na_2HPO_4 (pH 7.2), 500 mM NaCl, 1 mM EGTA, 10 mM β-mercaptoethanol, filtered) and passed at room temperature through an amylose affinity column (New England Biolabs, catalog #800-21) that was equilibrated with column buffer. The column was washed with 3 bed volumes of column buffer, 0.25% Tween 20 and with 3 bed volumes of column buffer. MBP-fetuin was eluted with 3 bed volumes of column buffer, 10 mM maltose. The A_{280} of the eluate was determined, and the material was stored at −20 °C.

Denaturation and Refolding of Recombinant Fetuin—The eluate from the amylose column was thawed and centrifuged to pellet precipitates. The solution was concentrated to A_{280} of approximately 4 with a Centricon 30 cartridge (Amicon), and the concentration of maltose was reduced to less than 1 mM with several rounds of concentrating the solution and rediluting in column buffer. The proteins were denatured and reduced by the addition of 40% (weight/volume) urea and 0.1 volume of 1 M dithiothreitol and nutated at room temperature for 2 h. The solution was centrifuged to remove precipitates, transferred into a syringe (piston removed), and slowly allowed to drip through a 27-gauge hypodermic needle into 1000 volumes of renaturation buffer (50 mM Tris (pH 8.0), 2 mM reduced glutathione, 0.2 mM oxidized glutathione, 0.01% sodium azide, filtered through a 0.2-μm filter). The buffer was slowly stirred in a siliconized Erlenmeyer flask at room temperature in the dark. Incubation was for 60 h.

The renatured protein was concentrated to 50 ml using an H1-P30-20 hollow fiber cartridge (Amicon) and then to 1.5 ml with a Centriprep 30 (Amicon). The solution was then subjected to 3 rounds of 10-fold dilutions in HBS (20 mM Hepes (pH 7.2), 150 mM NaCl) and subsequent reconcentrations to change the buffer to HBS. The solution was sterile-filtered and further concentrated in a Centricon 30 cartridge (sterilized with 70% ethanol and rinsed with sterile HBS). Protein concentration was determined with Bio-Rad BCA reagent and by estimation from the intensity of bands in acrylamide gels stained with Coomassie Blue. Protein concentrations up to 30 mg/ml could be achieved in this manner without protein precipitation.

Acrylamide gel electrophoresis (Novex) performed in the absence of reducing agents was used to assess the success of refolding of the MBP-fetuin preparations. Misfolded proteins tended to be cross-linked through intermolecular disulfide bridges, which could be identified on nonreducing gels. Western blots derived from nonreducing gels were probed with anti-MBP (New England Biolabs) or anti-fetuin antisera (Dako) to enhance sensitivity and to identify degradation products and contaminants. The MBP-fetuin preparations routinely were greater than 90% monomeric, with little multimeric or degraded MBP-fetuin and with little contaminating protein.

Osteogenesis in Rat Bone Marrow Cultures—Femoral bones were removed under aseptic conditions from adult male Wistar rats (120 g), cleaned of adherent soft tissues, and washed extensively in antibiotics. The distal ends were removed, and the marrow contents were flushed out with 10 ml of culture medium. The cells were dispersed by repeated passage through a 20-gauge needle and incubated in α- minimum Eagle's medium supplemented with 15% fetal bovine serum, ascorbic acid (50 μg/ml), antibiotics (penicillin G 100 μg/ml, gentamicin 50 μg/ml, Fungizone 0.3 μg/ml), 10 mM β-glycerophosphate, and vitamin C. The culture media were supplemented further with dexamethasone (10^{-8} M). After 6 days of culture, the cells were replated at a density of 1×10^2 cells/mm^2 in 96-well plates and grown for another 12–14 days, with changes of the same medium at 48-h intervals. At the end of the culture, the cells were fixed with 10% buffered formalin and stained for calcium with alizarin red S to identify mineralized bone nodules. To quantify mineralized tissue formation in the cultures, the absorbance at 525 nm was measured using a 96-well plate reader. Duplicate wells were stained for lipid content. Cells were fixed with 70% ethanol and stained with Sudan IV in acetone/ethanol for 20 min and then washed with 70% ethanol for photographic documentation.

Surface Plasmon Resonance—Binding constants for bovine serum fetuin and recombinant fetuin were measured using the BIAcore machine and BIA-evaluation software (Pharmacia Biosensor) (33, 34). TGF-β1 and BMP-2 were immobilized onto the carboxymethylated dextran surface of the CM5 sensor chip. The running buffer was 20 mM Hepes (pH 7.2), 150 mM NaCl, and the flow rate was 3 μl/min. Protein binding to the surface causes a change in reflected light, which is directly proportional to mass bound and is measured in arbitrary response units. Protein binding is observed in two ways, increasing response with time during the injection phase and the difference in the position of the base line before and after injection. Regeneration of the surface to remove bound analyte was done by injecting 10 μl of 20 mM NaOH.

RNA Isolation and Northern (RNA) Blot Analysis—RNA was extracted from the rat bone marrow cultures by using a guanidinium isothiocyanate procedure as described (20). Total RNA was run on a 1% agarose gel containing 2% formaldehyde and 1 × MOPS buffer and transferred to Genescreen (Dupont) membrane. Full-length rat cDNA probes for AP, OPN, BSP, and collagen type I were labeled using [^{32}P]dCTP by random priming and used to probe Northern blots. Hybridization was done at 65 °C using a sodium phosphate hybridization buffer (0.5 M NaP, 0.001 M EDTA, 7% SDS). The membrane was washed for 15 min at room temperature followed by 2 × 30 min at 65 °C (30 mM NaP, 0.1%SDS) before exposure.

RESULTS

Commercially available sources of bovine fetuin are purified from serum by differential precipitation methods. It has been suggested in the past that impurities in fetuin preparations might contribute to some of the effects reported for fetuin in biological assays (reviewed in Ref. 4). We produced recombinant fetuin as an MBP fusion protein in *E. coli* to confirm cytokine binding activity and anti-osteogenic activities attributed to native fetuin. Following affinity purification and refolding *in vitro*, MBP-fetuin was more than 90% monomeric with a minor fraction of dimeric protein as indicated by mobility in nondenaturing SDS-PAGE (Fig. 1). Surface plasmon resonance measurements showed that MBP-fetuin and native fetuin protein bound to TGF-β1 with similar on- and off-rates (Table I). MBP-fetuin also bound to BMP-2; however, the off-rate was faster than that of native fetuin binding to BMP-2. Neither denatured MBP-fetuin nor reduced and alkylated native fetuin bound to TGF-β cytokines (data not shown). These results establish that recombinant MBP-fetuin binds TGF-β cytokines with affinities similar to those of commercially prepared fetuin.

Treatment of RBMC with 10^{-8} M dex for 12 days resulted in an increase in active TGF-β in the culture medium (12, 21) and in osteogenesis and mineralization. TGF-β1 is required for osteogenesis in the dex-RBMC cultures, because specific TGF-β antagonists like sTβRII/Fc and polyclonal anti-TGF-β1 antibody blocked mineralization. sTβRII/Fc showed an IC_{50} value of 300 pM in the dex-RBMC assay (Fig. 2). MBP-fetuin and native fetuin proteins showed IC_{50} values of 2–3 μM in dex-RBMC (Fig. 2). These values are very similar to the fetuin-TGF-β1 binding constant measured by surface plasmon resonance (Table I). The control proteins, human transferrin and bovine serum albumin, had no effect. These results confirmed that fetuin and not a serum contaminant of the commercial preparations is the active molecule in the dex-RMBC assay.

Exogenous TGF-β1 at concentrations greater than 10 pM also completely inhibited mineralization (Fig. 3*A*). Therefore, it appears that response to TGF-β1 is biphasic, and both cytokine and fetuin may promote or antagonize osteogenesis depending upon their relative concentrations. To test this hypothesis, fetuin and anti-TGF-β1 antibodies were titrated into cultures with either 0.3 or 10 pM of exogenous TGF-β1. Indeed, both TGF-β antagonists completely reversed the inhibitory effect of 10 pM TGF-β1 and also suppressed the action of low dose TGF-β (Fig. 3, *B* and *C*). In effect, optimal osteogenesis in the dex-RBMC cultures is dependent upon the ratio of TGF-β to antagonist protein.

Time course experiments were done to determine whether inhibition of osteogenesis in dex-RBMC by fetuin occurs during osteoblast differentiation or late with hydroxyapatite formation. Inhibition by fetuin occurred during the first 6 days of culture, well before mineralization, which is observed at 10–12 days (Fig. 4). Furthermore, when added after 6 days of culture, 1–30 μM concentrations of fetuin did not inhibit mineralization. Fetuin has been shown to inhibit hydroxyapatite formation *in vitro*, with an IC_{50} of 0.5 μM, via a calcium binding motif in the first cystatin-like domain of the protein (22); however, in our hands this activity did not inhibit mineralization in the RBMC cultures.

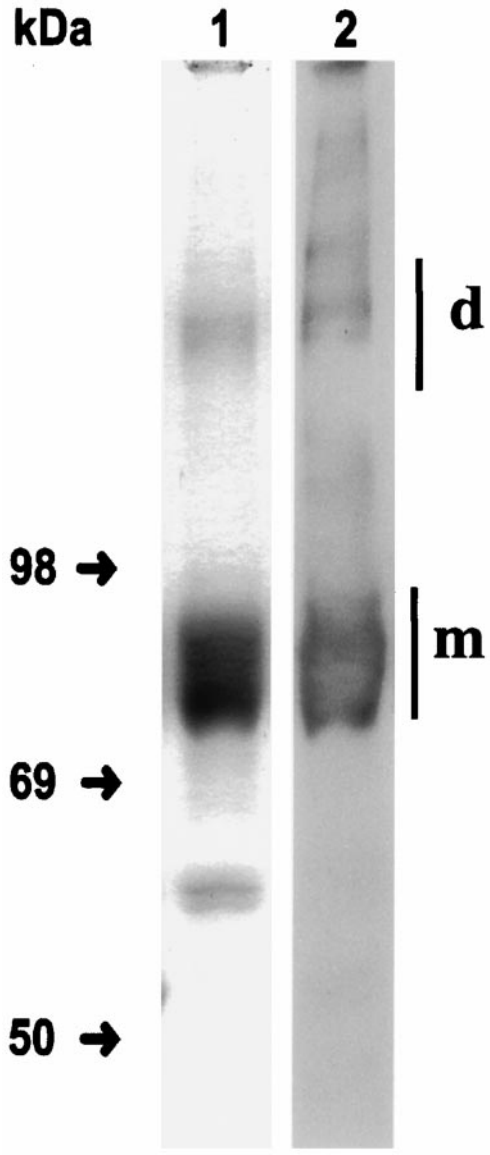

FIG. 1. **Recombinant MBP-fetuin is pure and predominately monomeric.** MBP-fetuin fusion protein produced in *E. coli* was purified and refolded as described under "Experimental Procedures." The protein (10 μg) was separated by nonreducing SDS-PAGE stained with Coomassie Blue (*lane 1*). Western blotting was performed with rabbit anti-fetuin antiserum (*lane 2*). The majority of the protein migrated as a monomer (*m*), and less than 10% had the expected molecular weight for the dimer (*d*).

To further characterize the developmental stage at which fetuin and high dose TGF-β1 block osteogenesis in dex-RBMC, transcripts of several genes normally induced during osteogenic differentiation were examined by Northern blot analysis. Transcript levels for AP, BSP, OPN, and Coll I increased 10 days after the addition of dex to the RBMC cultures (Fig. 5). Fetuin and high dose TGF-β1 suppressed dex-induced increases in these transcripts, whereas anti-TGF-β1 antibodies had no effect. This suggests that fetuin and high dose TGF-β1 both block early stages of differentiation, whereas anti-TGF-β1 inhibits at a later stage, following progenitor cell commitment. The results suggest that TGF-β cytokines are required at more than one stage of cellular differentiation and that TGF-β1 is specifically required following induction of AP, BSP, OPN, and Coll I gene expression.

TABLE I

TGF-β1 and BMP-2 binding to fetuin and recombinant MBP-fetuin

Binding constants for fetuin and recombinant MBP-fetuin binding to TGF-β and BMP-2 measured using the BIAcore and BIA-evaluation software (Pharmacia Biosensor). The change in response with time was plotted for each analyte concentration (ie. dR/dT *vs.* R) and the slopes of these lines were then plotted as a function of analyte concentration (*i.e.* d(dR/dT)/dR *vs.* C), where the slope yields k_{ass}. The dissociation rate constant, k_{diss}, was obtained after analyte injection was discontinued as the slope of $\ln(R_{t1}/R_{tn})$ *vs.* time. K_D values are k_{diss}/k_{ass}. Variance for k_{ass} is the standard error of the linear regression plot and for k_{diss} values, are the mean ± range of three or more independent injections.

Ligand	Analyte	k_{ass}	k_{diss}	K_D
		$M^{-1}\ s^{-1}$	s^{-1}	M
TGF-β1	Fetuin	$8.7 \times 10^2 \pm 2.7 \times 10^2$	$1.9 \times 10^{-3} \pm 6.0 \times 10^{-4}$	2.2×10^{-6}
TGF-β1	MBP-fetuin	$8.1 \times 10^2 \pm 7.5 \times 10^1$	$4.8 \times 10^{-3} \pm 5.4 \times 10^{-4}$	5.9×10^{-6}
BMP-2	Fetuin	$2.4 \times 10^3 \pm 1.2 \times 10^3$	$6.5 \times 10^{-5} \pm 2.1 \times 10^{-5}$	2.7×10^{-8}
BMP-2	MBP-fetuin	$9.0 \times 10^3 \pm 6.0 \times 10^2$	$3.9 \times 10^{-3} \pm 9.9 \times 10^{-4}$	4.3×10^{-7}

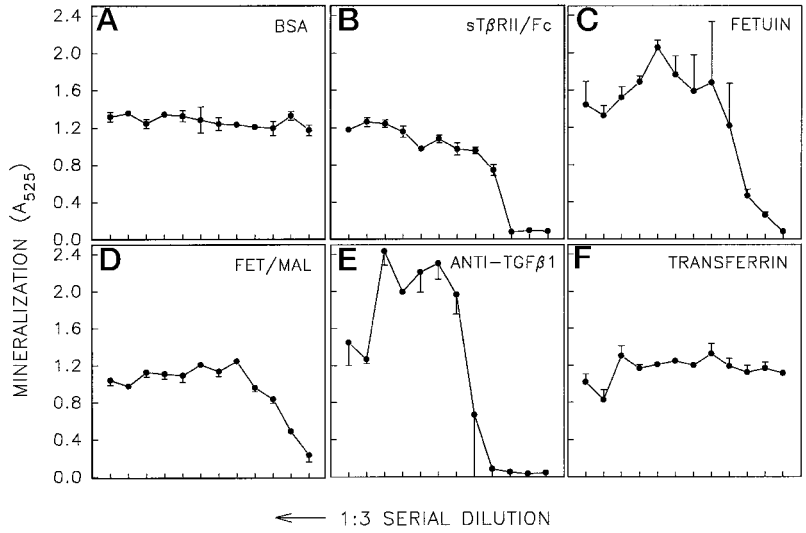

FIG. 2. **Inhibition of mineralization in dex-RBMC.** Agents were added to the cultures in 1/3 serial dilutions beginning at the following concentrations: *A*, bovine serum albumin (*BSA*), 15 μM; *B*, sTβRII/Fc, 13 nM; *C*, commercial fetuin, 30 μM; *D*, recombinant MBP-fetuin (*FET-MAL*), 12.5 μM; *E*, anti-TGF-β1 antibodies, 13.3 μM; *F*, transferrin, 13.2 μM. The first data point in each *panel* represents control with no inhibitor.

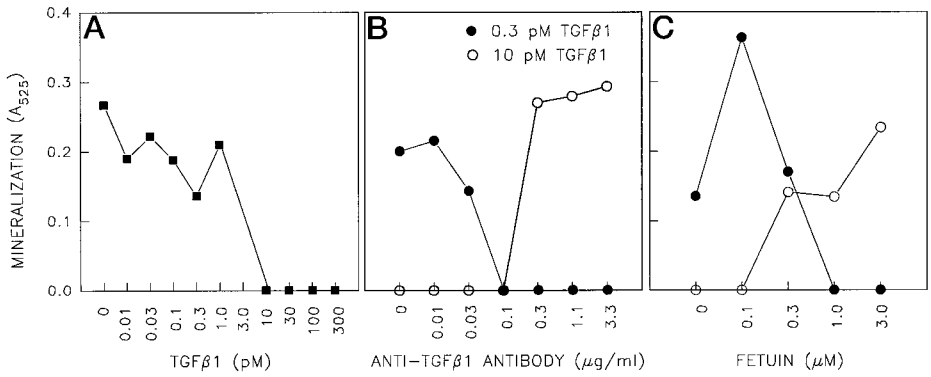

FIG. 3. **Biphasic response to TGF-β1 and antagonist proteins in dex-RBMC.** *A*, response to exogenous TGF-β1 showing that ≥10 pM of exogenous TGF-β1 completely suppressed mineralization. TGF-β1 in the dex-RBMC is less than 1 pM. *B*, titration of neutralizing anti-TGF-β1 antibody into cultures treated with either 0.3 pM (*closed*) or 10 pM (*open*) exogenous TGF-β1. *C*, titration of bovine fetuin into cultures treated with either 0.3 pM (*closed*) or 10 pM (*open*) exogenous TGF-β1. The first data point in each *panel* has no inhibitor.

The inhibition of osteogenesis in dex-RBMC by fetuin was accompanied by the appearance of large cells in the cultures. These cells stained positive with Sudan IV, a characteristic of adipocytes (Fig. 6*A*). A reciprocal relationship between Sudan IV-positive cells and alizarin red S staining cultures was observed with increasing fetuin concentrations in the dex-RBMC (Fig. 6*B*). Sudan IV-positive cells were also observed in the high dose TGF-β-treated cultures but at a much lower frequency (*i.e.* 5–10/well) than in fetuin-treated cultures. Cultures treated with anti-TGF-β1 antibody showed no evidence of Sudan IV-positive cells or mineralization, consistent with a block at a stage following commitment to an osteogenic lineage but before terminal differentiation and mineralization.

DISCUSSION

Both fetuin and TGF-β1 are present in bone matrix at high concentrations relative to other tissues and have previously been implicated in bone remodeling (1, 2, 23). The TRH1 peptide domain of fetuin is related in sequence to the cytokine binding domain of TβRII, and fetuin binds directly to TGF-β1, -β2, and BMP-2, -4, -6 cytokines (12). In this report, we showed that recombinant MBP-fetuin produced in *E. coli* and refolded *in vitro* has similar binding kinetics for TGF-β1 and BMP-2 as the native protein from bovine serum. Therefore, fetuin requires neither mammalian glycosylation nor phosphorylation to bind TGF-β cytokines, as these modifications are absent in

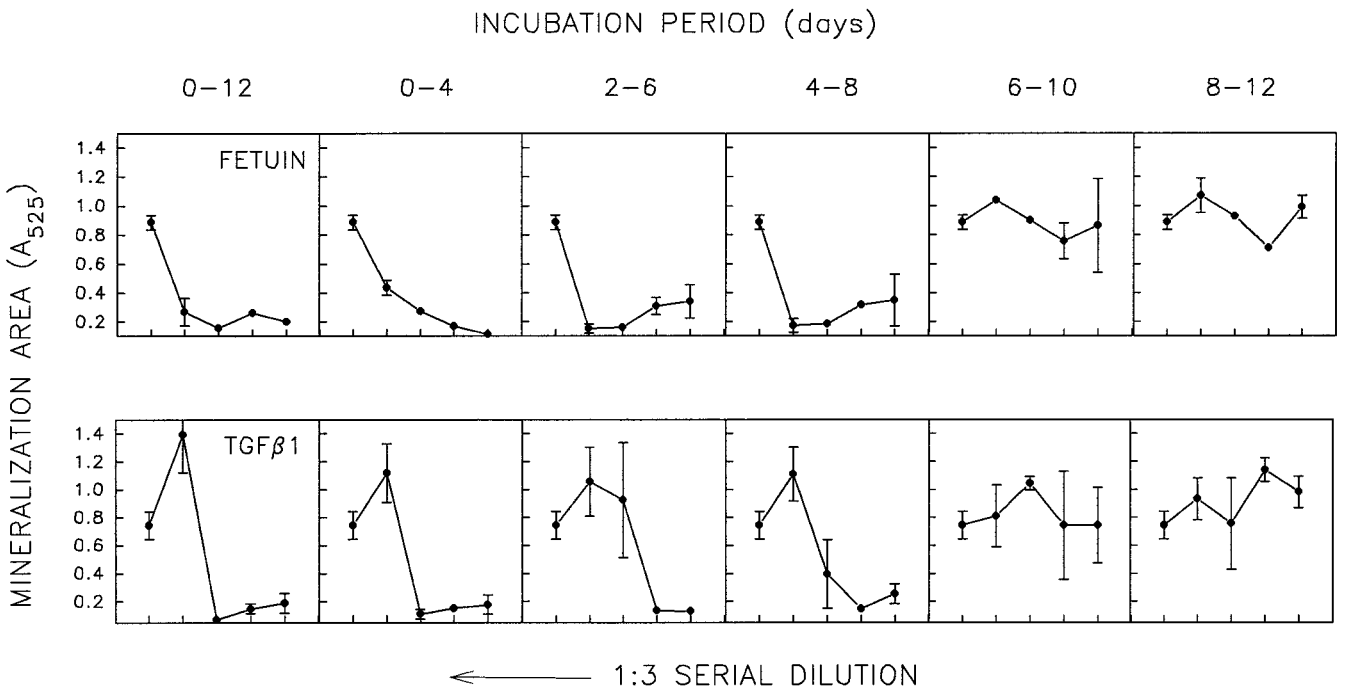

FIG. 4. **Inhibition of osteogenesis by fetuin and high dose TGF-β1 occurs during the first 6 days of culture.** Each *panel* shows a 1/3 titration series of the inhibitor from right to left, with no inhibitor on the left of each *panel*. The time period during the culture that inhibitors were present is indicated on the *upper axis*. The *top* and *bottom rows* are time and dilution series for fetuin (30 μM) and TGF-β (100 pM), respectively.

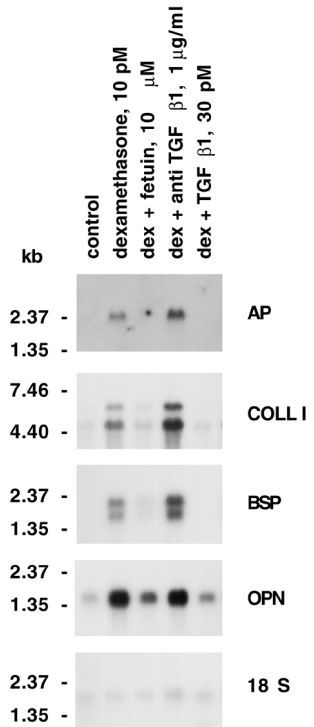

FIG. 5. **Expression of bone-associated transcripts in dex-RBMC.** Northern blot detection of AP, Coll I, BSP, and OPN in 10-day rat bone marrow cultures treated as indicated above each *lane*. High dose TGF-β1, fetuin, and anti-TGF-β1 antibodies blocked mineralization as confirmed by alizarin red S staining in parallel cultures on day 12.

the *E. coli*-produced protein. In addition to comparable affinity for cytokines, recombinant and native fetuin had similar IC_{50} values for inhibition of osteogenesis in dex-RBMC, confirming that fetuin rather than a contaminant of the native protein preparation was the active molecule in the assay.

Titration of TGF-β1 into dex-RBMC produced a biphasic response with complete inhibition at ≥10 pM and an optimal osteogenic response at ~1 pM. By titrating both TGF-β1 and antagonist proteins into the dex-RBMC, we have demonstrated that the optimal osteogenic response is a function of their relative concentrations. The working range for inhibition observed in the dex-RBMC cultures was 0.3 to 10 pM TGF-β and 0.1 to 3 μM fetuin, a molar ratio of 1/300,000 and a reflection of the physiological levels of these proteins. The biphasic response to TGF-β1 may be a manifestation of cytokine and antagonist gradients *in vivo*, creating a zone or front of osteogenic differentiation defined by opposing gradients of cytokine and antagonist. Both fetuin and TGF-β1 levels are more concentrated in bone rather than in osteoid and marrow (24, 25). Hence, osteoclast-mediated destruction of bone during remodeling may increase local TGF-β cytokine concentrations, whereas fetuin and other serum antagonists may serve to confine the zone of precursor cell recruitment and osteogenic differentiation. A number of TGF-β/BMP/activin cytokine-binding proteins with antagonist activity have been identified in mammals, *Xenopus* and *Drosophila* (13). In the gastrula stage, the long range effects of two BMP inhibitors, noggin and chordin, establish a BMP-4 gradient (26). Mice lacking noggin protein show failure of chondrocyte positioning to form joints (27). Other TGF-β cytokine-binding proteins include follistatin, serum α2-macroglobulin (28), soluble betaglycan (29), the proteoglycans, decorin, biglycan, and fibromodulin (30;31), each with varying cytokine specificity and affinity. Biglycan is implicated in bone remodeling, as mice deficient in biglycan show a progressive deficiency in bone mass with age (32). Fetuin-deficient mice also show differences in bone properties.[2]

Bone loss and risk of fractures are common side effects of glucocorticoid treatment of rheumatoid arthritis, chronic active hepatitis, and asthma. Glucocorticoids stimulate osteogenesis and mineralization in RBMC, but repeated stimulation results in depletion of osteoprogenitor cells, which may also contribute to bone loss *in vivo* (33). Glucocorticoids enhance TGF-β1 levels in RBMC cultures and may do so *in vivo* as well, but levels of cytokines and antagonists *in vivo* likely differ from cell culture

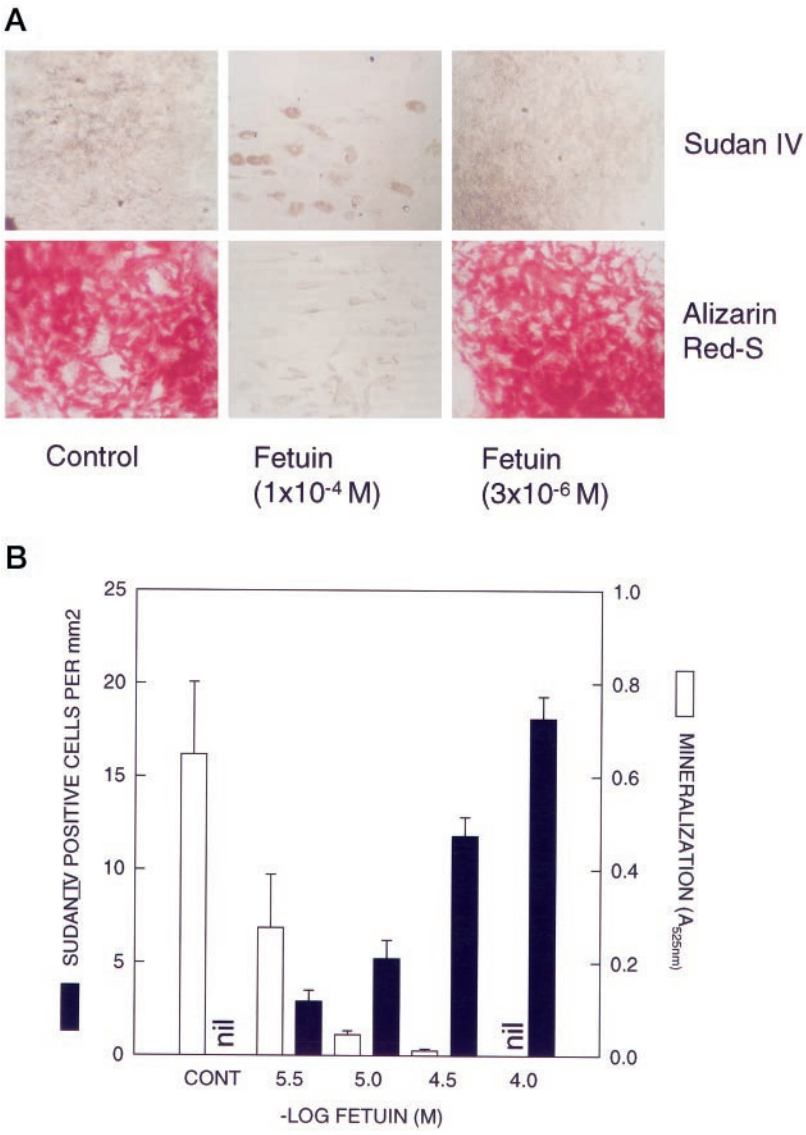

FIG. 6. **Inhibition of osteogenesis by fetuin induces adipocyte-like cells.** *A*, dex-RBMC-treated with 0, 1×10^{-4} M, or 3×10^{-6} M fetuin. After 12 days of culture, duplicate wells were stained with either Sudan IV for lipid content or alizarin red S for calcium phosphate deposition. *B*, numeration of Sudan IV-positive cells and alizarin red S staining in RBMC cultures with increasing concentrations of fetuin.

experiments. More importantly, the biphasic osteogenic response to TGF-β1 suggests that either overexpression or depletion of the cytokine could suppress osteogenesis *in vivo*. In this regard, TGF-β1-deficient mice show decreased longitudinal growth and reduced bone mass (34), and TGF-β2 transgenic mice expressing the cytokine in osteoblasts also show loss of bone mass and increased mineral apposition rates (35). Although the gross features of bone morphology in these mice are similar, the molecular and cellular phenotypes associated with overexpression and depletion of TGF-β in bone likely differ substantially. Nevertheless, with a biphasic osteogenic response to TGF-β1, it is possible that both depletion and increased availability of cytokine have similar pathological consequences on bone remodeling. The levels of antagonist proteins in the microenvironment and their effects on the strength of cytokine gradients may determine the rates and spatial precision of osteogenesis in remodeling bone. Although opposing gradients of factor and antagonist reduce the potency of a signal, they can effectively increase both the sharpness of the concentration gradient, and in addition, the alignment between the sources of cytokine and antagonist may improve directionality of cell movement (36). In bone remodeling, this feature of cytokine regulation may add precision and sharpness to the boundaries between osteoid, mineralized bone and non-bone regions.

Osteogenic differentiation in dex-RMBC involves sequential events, including cell proliferation, commitment of pre-osteoblast cells, matrix deposition, and finally matrix mineralization (18). The addition of fetuin or anti-TGF-β1 antibodies to dex-RBMC before the 6th day of the 12-day culture period was necessary and sufficient to block mineralization. This indicates that both proteins interfere with osteogenic differentiation and act before the mineralization stage. However, fetuin appeared to block bone formation at an earlier stage of differentiation than anti-TGF-β1 antibodies, as the latter protein did not block induction of the bone-associated transcripts AP, BSP, OPN, and Coll I. Tamoxifen, an estrogen analogue used in the treatment of breast cancer, also blocks dex-RBMC osteogenesis at a stage post-expression of the AP, BSP, OPN, and Coll I transcripts (19). Tamoxifen treatment reduces bone loss, possibly by inducing TGF-β gene expression (19).

Fetuin binds to BMPs as well as the TGF-β cytokines and therefore may block osteogenesis earlier by neutralizing several cytokines. The addition of BMPs to RBMC enhances osteogenesis (37). Similarly, glucocorticoid stimulates BMPs- 2, 4, 5, 6, and 7 production in cultured fetal calvarial cell, which contribute to osteogenic commitment *in vitro* (38). Osteogenesis in dex-RBMC proceeded further in the presence of anti-TGF-β1 antibodies, suggesting the cytokine was specifically required in the later phases of matrix deposition and mineralization. In

mouse bone marrow cultures, TGF-β1 levels have been shown to rise in the first 4–5 days of osteogenic differentiation, then decline and rise again late in the mineralization phase (39). The early increase in TGF-β1 appears to coincide with the proliferation phase of pre-osteoblast cells and may also be required for osteogenesis. However, the addition of high dose TGF-β1 into the cultures suppressed dex-induced expression of the bone-associated genes. TGF-β1 is known to suppress proliferation of many cell types and, if present at high concentration or at the wrong time, may block expansion of the osteoblast precursor cells.

Dex-RBMC differentiate primarily along the osteogenic lineage under the culture conditions used in our experiments, but other cell lineages including macrophage and adipocyte have also been observed in RBMCs (40). Inhibition of osteogenesis by either fetuin and, to a lesser degree, high dose TGF-β1 resulted in an increase in adipocyte-like cells in the cultures, suggesting that TGF-β regulates a reciprocal relationship between osteogenic and adipogenic differentiation in the cultures. Consistent with this interpretation of the results, fetuin-deficient mice show a marked increase in marrow adipocytes.[2] Our results show that fetuin functions as a TGF-β/BMP cytokine antagonist and regulator of osteogenesis and suggests that overlapping gradients of cytokines and antagonists regulate osteogenesis in bone remodeling.

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