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Fetuin/α2-HS Glycoprotein Is a Transforming Growth Factor-β Type II Receptor Mimic and Cytokine Antagonist*

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The serum glycoprotein fetuin is expressed during embryogenesis in multiple tissues including limb buds and has been shown to promote bone remodeling and stimulate cell proliferation *in vitro*. In this report, we demonstrate that fetuin antagonizes the antiproliferative action of transforming growth factor-β1 (TGF-β1) in cell cultures. Surface plasmon resonance measurements show that fetuin binds directly to TGF-β1 and TGF-β2 and with greater affinity to the TGF-β-related bone morphogenetic proteins (BMP-2, BMP-4, and BMP-6). In a competitive enzyme-linked immunosorbent assay, fetuin blocked binding of TGF-β1 to the extracellular domain of TGF-β receptor type II (TβRII), one of the primary TGF-β-binding receptors. A comparison of fetuin and TβRII shows homology in an 18–19-amino acid sequence, which we have designated TGF-β receptor II homology 1 domain (TRH1). Since the TRH1 sequence is known to form a disulfide loop in fetuin, cyclized TRH1 peptides from both fetuin and TβRII were chemically synthesized and tested for cytokine binding activity. Cyclized TRH1 peptide from TβRII bound to TGF-β1 with greater affinity than to BMP-2, while the cyclized TRH1 peptide from fetuin bound preferentially to BMP-2. Finally, fetuin or neutralizing anti-TGF-β antibodies blocked osteogenesis and deposition of calcium-containing matrix in cultures of dexamethasone-treated rat bone marrow cells. In summary, these experiments define the TRH1 peptide loop as a cytokine-binding domain in both TβRII and fetuin and suggest that fetuin is a natural antagonist of TGF-β and BMP activities.

Fetuin was first identified over 5 decades ago as a major protein component of bovine fetal serum (1), but its biological function has remained unclear. Bovine fetuin is a globular 341-amino acid protein with six internal disulfide bonds and three *N*-linked and two *O*-linked oligosaccharide chains (2, 3). Primary amino acid sequence and the position of cysteine residues are well conserved in human, bovine, sheep, rat, and mouse fetuins (2, 4–6). Fetuin levels in human plasma are regulated in the manner of a negative acute phase reactant (7), and the inflammation-associated cytokine IL-1 has been shown to suppress fetuin transcript levels in cultured hepatocytes (8). α2-HS glycoprotein, the human homolog of fetuin, is secreted by adult liver into the peripheral circulation and accumulates to high levels in bone (9, 10). Fetuin also appears to be expressed in bone, as transcripts have been detected in chondrocytes and osteoblasts (11). During mouse embryogenesis, fetuin mRNA is expressed in a number of developing tissues and organs, including the heart, lung, kidney, nervous system, and liver (12). In addition, fetuin mRNA is expressed in the developing limb buds of 12-day mouse embryos but not at 16 or 19 days of gestation (12). The biological effects reported for fetuin in cell culture appear in many respects to oppose those described for transforming growth factor-β (TGF-β)[1] cytokines. Specifically, fetuin promotes cell growth in tissue culture (13), enhances bone resorption (14), and stimulates adipogenesis in cell culture models (15). In this report, we have examined the possibility that fetuin is a TGF-β cytokine antagonist.

The TGF-β superfamily of cytokines has more than 30 members, and most have been grouped into the TGF-β, BMP/DVR, and activin subfamilies (16, 17). The TGF-β subgroup has been shown to induce extracellular matrix production and differentiation in many cell types and to inhibit epithelial and lymphoid cell proliferation by down-regulating the activity of cyclin/Cdk kinase complexes (18, 19). TGF-β cytokines are present in bone matrix at concentrations approximately 100-fold higher than in soft tissues (20) and are required for the formation of mineralized extracellular matrix *in vitro* (21, 22). The BMP proteins possess osteoinductive activity, stimulating presumptive mesenchymal progenitor cells to differentiate into chondrocytic and osteocytic lineages (22, 23). BMPs are expressed during embryogenesis (24) and their importance in skeletal development is evident from studies of null mutations in mice that result in multiple skeletal abnormalities (25).

TGF-β cytokines are assembled as disulfide-linked dimers, secreted in a precursor or latent form, and subsequently become activated by protease cleavage and release of the active cytokine (16). Plasma TGF-β1 levels in humans are 100-1000-fold greater than required for many cellular responses, suggesting a large fraction is latent or inactivated by binding to serum constituents (20). A number of TGF-β-binding glycoproteins in serum and tissues have been identified and may play a role in regulating cytokine availability. α2-Macroglobulin (26); soluble betaglycan (27); and the proteoglycans decorin, bigly-

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; TβRI and TβRII, TGF-β receptor type I and II, respectively; BMP, bone morphogenetic protein; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; ELISA, enzyme-linked immunosorbent assay; ActRII, activin receptor type II.

can, and fibromodulin have been shown to bind TGF-β cytokines (28, 29). Follistatin has been shown to bind and antagonize the activity of activin (30). A null mutation in the follistatin gene resulted in widespread defects in muscle, skin, and skeleton, showing some phenotypic overlap with the activin null mutation (31).

The TGF-β signaling receptors have been grouped into type I and type II subclasses; both types are transmembrane Ser/Thr kinases (32). Signaling by TGF-β or activin is initiated by cytokine binding to type II receptors (*e.g.*, TβRII, ActRII, or ActRIIB), which recruits and phosphorylates the type I receptors (*e.g.*, TβRI or ActRI). Activated type I receptors then phosphorylate as yet unidentified downstream signaling components (32).

Herein, we report that fetuin binds to and antagonizes the antiproliferative and osteogenic activity of TGF-β cytokines *in vitro*. It is further shown that type II cytokine receptors and fetuin share sequence homology in a domain designated TRH1. Cyclized TRH1 peptides from fetuin and TβRII bound directly to cytokine and antagonized TGF-β binding to TβRII, suggesting that TRH1 is a cytokine-binding domain of the receptor. In cell culture, fetuin blocked TGF-β activity with an IC_{50} that was similar to both the K_D measured by surface plasmon resonance and the physiological concentrations for fetuin in bone and serum. These experiments define the TRH1 peptide loop in TβRII and fetuin as a cytokine-binding domain and suggest a function for fetuin as a natural antagonist of TGF-β/BMP activities.

EXPERIMENTAL PROCEDURES

Materials—Bovine fetuin was purchased from Sigma (catalog number F3004), adult human fetuin (α2-HS glycoprotein) from Calbiochem, and BSA (fraction V) from Boehringer Mannheim. Bovine fetuin is purified by ammonium sulfate fractionation of fetal calf serum followed by gel filtration chromatography. Human fetuin was purified from adult human serum by sequential runs of two DEAE columns, followed by affinity chromotography, gel filtration, and HPLC on a Mono Q column. Human TGF-β1 was from Collaborative Research (Bedford, Ma.). Recombinant human TGF-β2, BMP-2, BMP-4, and BMP-6 were kindly supplied by the Genetics Institute (Boston, MA).

TGF-β Inhibition of Cell Proliferation—Mv1Lu epithelial cells were seeded at 20,000 cells/well in a 96-well plate containing 0.2% fetal calf serum in α-minimal essential medium and incubated with increasing concentrations of fetuin or BSA for 18 h. The cells were then pulsed with 8 μCi/ml [*methyl*-^{3}H]thymidine (DuPont NEN) for 6 h, trypsinized, harvested onto filter mats, and counted in a β-counter.

Surface Plasmon Resonance—Binding constants for fetuin and TRH1 peptide interactions with TGF-β and BMPs were measured using the BIAcore machine and BIA-evaluation software (Pharmacia Biotech Inc.) (33, 34). TGF-βs and BMPs 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.

Peptides—Peptides were synthesized on phenylacetoamidomethyl resin support using *t*-butoxycarbonyl amino acids and an automated peptide synthesizer. The peptides were extracted from the resin with 10% aqueous acetic acid, lyophylized, and purified by reverse phase HPLC. The TRH1 domain in fetuin is at amino acid positions 114–132, and in TβRII at positions 84–101 of the open reading frames. Synthetic peptides of 20 amino acids corresponding to TRH1 of bovine fetuin and human TβRII were made, CDIHVLKQDGQFSVLFTKCD and CVAVWRKNDENITLETVCHD, respectively. The peptide CKGSVIQKALGGEDVRVTCT in fetuin, corresponding to amino acids 230–249 (*i.e.* loop 2) was also synthesized as a control.

To cyclize the peptides, samples were reduced with 5 mM dithiothreitol and then brought to a final concentration of 60 μM in 25 mM ammonium acetate (pH 8.5). After stirring in the dark for 30 min at 20 °C with 30 mM potassium ferricyanide, the peptide was mixed with AG3-X4A resin (Bio-Rad), filtered, lyophilized, and desalted on a 50 × 2.5-cm column of Biogel P2 (Pharmacia) developed in water and further purified by reverse phase HPLC. Ion spray mass spectroscopy confirmed cyclization and lack of interpeptide cross-linking.

A search of the PIR protein data bank was done to identify proteins with putitive TRH1 domains. Conserved residues in the TRH1 domains of fetuin, TβRII, and activin type II receptor were aligned visually and used to search the PIR protein data bank with the pattern, C*X*{0,1}(V,I,F,Y)*X*{1,3}(V,W,L)*X*{0,1}(K,R)*X*{4,5}(V,I,F)*X*{1,2}(L,I)*X*{2,4}-C*X*{0,2}D, using the the GCG routine "findpatterns."

Competition of TGF-β Binding to Type II Receptor—The Quantikine ELISA kit (R & D Systems) for measurement of TGF-β is supplied as 96-well plates precoated with the extracellular domain of human TβRII. To study competition of TGF-β1 binding, the cytokine was brought to a final concentration of 40 pM in diluent provided with the kit, either alone or with the antagonist fetuin (400 μM), TRH1 peptide from TβRII (200 μM), or BSA (400 μM) and incubated for 1.5 h at 20 °C in siliconized Eppendorf tubes with shaking. This was followed by a 5-min incubation with the TβRII in coated ELISA wells with shaking, washing as recommended, and a 1-h incubation with polyclonal antibody against TGF-β1 conjugated to horseradish peroxidase. Plates were developed and optical density was read at 450 nm.

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-minimal essential medium supplemented with 15% fetal bovine serum, ascorbic acid (50 μg/ml), antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamicin, 0.3 μg/ml fungizone) and 10 mM β-glycerophosphate. The culture media were supplemented further with dexamethasone (10^{-8} M) and various concentrations of fetuin. Following 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 in the same medium. At the end of culture, the cells were fixed and stained for calcium with alizarin red-S to identify mineralized bone nodules. To quantify mineralized tissue formation in the cultures, morphometric measurements of the bone nodules, reported as mineralized area (mm^2/well) were carried out with the aid of the Bioquant IV system (Bioquant, Nashville, TN) using a Leitz Orthoplan microscope. TGF-β levels in culture supernatants taken 4 days prior to scoring mineralization were measured with and without heat activation (80 °C for 10 min) using the Mink cells as the indicator cell line as described above. Neutralizing anti-TGF-β antibodies supplied by R & D sytems were specific for TGF-β1, with 100-fold lower reactivity for TGF-β2 and TGF-β3.

RESULTS

Fig. 1 shows a dose response for neutralization of TGF-β1 activity by bovine fetuin in cell culture. Mv1Lu lung epithelial cells are highly sensitive to the antiproliferative action of TGF-β1, showing an IC_{50} of 8×10^{-12} M. Fetuin blocked the antiproliferative activity of exogenously added TGF-β1, with an IC_{50} of approximately 1×10^{-6} M. Saturation with fetuin in this assay showed greater than 100% neutralization, suggesting that fetuin may be neutralizing endogenous TGF-β produced in the cultures (Fig. 1*A*). Indeed, both fetuin and neutralizing monoclonal anti-TGF-β1–3 antibody stimulated Mv1Lu cell proliferation in the absence of exogenous TGF-β (Fig. 1*B*). The growth-promoting effects of fetuin and anti-TGF-β antibodies were not additive at near saturating levels of fetuin, suggesting that both proteins stimulate cell growth by binding to and antagonizing TGF-β (data not shown).

As shown in Fig. 1*B*, Mv1Lu cell growth was also inhibited by BMP-2 with an IC_{50} of $1–2 \times 10^{-8}$ M. Fetuin blocked the antimitogenic activity of BMP-2 with 50% neutralization at approximately 6×10^{-7} M (Fig. 1*B*). Although the specific receptors on Mv1Lu cells that mediate responses to BMP-2 have not been determined, BMP-2 can be cross-linked to putative cytokine receptors on Mv1Lu cells.[2]

[2] J. Wrana, personal communication.

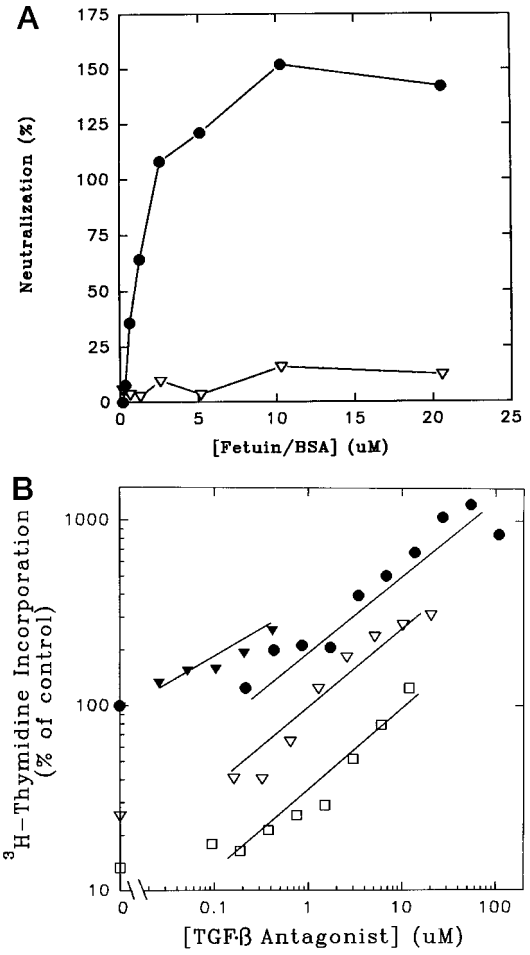

FIG. 1. **Fetuin neutralizes the anti-proliferative activity of TGF-β and BMP-2 in cultures of Mv1Lu cells.** *A*, fetuin (●) or BSA (▽) was titrated as indicated on the *x*-axis into cultures of Mv1Lu cells, in the presence of 8×10^{-12} M human TGF-β1. TGF-β1 neutralization is expressed as percentage of the control with no TGF-β1 added. *B*, titration of fetuin in the presence of 1.2×10^{-11} M TGF-β1 (□) and 3.3×10^{-8} M BMP-2, (▽). Titration of fetuin (●) or neutralizing monoclonal anti-TGF-β1–3 antibody (▼) in the absence of added cytokine are also shown. Each point represents the mean of duplicate determinations, and the results are representative of three experiments.

To examine whether fetuin inhibits TGF-β1 and BMP-2 activity by binding directly to the cytokine, association (k_a) and dissociation (k_d) rates for fetuin binding to TGF-β cytokines were measured by surface plasmon resonance (33). Purified cytokines were covalently coupled to a carboxymethylated dextran surface, and binding molecules (*e.g.*, fetuin) were passed in a fluid phase across the surface. Fig. 2*A* shows overlay plots for bovine fetuin binding to immobilized BMP-2. Fetuin bound specifically to immobilized TGF-β1, TGF-β2, BMP-2, BMP-4, and BMP-6 with varying affinities, whereas BSA and transferrin did not bind to these cytokines (Table I). The dissociation constant (K_D) for fetuin-TGF-β1 binding was 2.2×10^{-6} M, a value similar to the IC_{50} for neutralization of TGF-β1 by fetuin (Fig. 1). The affinity of bovine fetuin was 100-fold greater for BMP-2 than for TGF-β1. Human fetuin/α2-HS glycoprotein, which has 65% amino acid identity with bovine fetuin, bound to BMP-2 with a K_D similar to that of bovine fetuin (Table I). The affinity of cytokine binding to bovine fetuin was BMP-2 > BMP-4 > BMP-6 > TGF-β1 > TGF-β2, a rank order that correlates with the degree of sequence divergence from BMP-2 (Table I).

To explore the possibility that fetuin is an antagonist of receptor binding by virtue of a shared cytokine-binding domain, the bovine fetuin amino acid sequence was compared with that of the TGF-β receptors. After aligning cysteine residues, we noted a region of similarity flanked by cysteines in fetuin (19 amino acids) and TGF-β type II receptors (18 amino acids), which we have designated TGF-β receptor II homology 1 domain (TRH1) (Fig. 3*A*). The TRH1 domain is present in TβRII, ActRII, ActRIIb, and BMP-RII but absent in the type I receptors (32, 35–38). The TRH1 sequences in fetuin and TβRII share 41 and 65% amino acid identity and similarity, respectively. A low stringency search of the PIR protein data bank was done using conserved residues in the TRH1 domains of fetuin and the type II receptors for TGF-β and activin (see "Experimental Procedures"). The search yielded 32 matches, 25 of which represented fetuin ($n = 5$), TβRII ($n = 2$), and type II activin receptors ($n = 18$) from various species. The search algorithm did not identify TRH1 sequences in several other cytokine-binding proteins including α2-macroglobulin, betaglycan, decorin, and follistatin.

The intramolecular disulfide bonds in human fetuin have previously been determined (3), and the TRH1 domain forms a peptide loop. Algorithms for secondary structure predict a β-sheet-turn-β-sheet structure for the TRH1 peptides of both fetuin and TβRII (Fig. 3*A*). Low ultraviolet CD spectra analysis of cyclized synthetic TRH1 peptide from TβRII in water showed 58% β-sheet, 42% coil, confirming this prediction (Fig. 4). In the presence of the α-helix-inducing solvent 2,2,2-trifluroethanol (75%), β-sheet content remained similar, and coil was reduced at the expense of 20% α-helix. These data suggest that the looped peptides form stable secondary structure in aqueous solution.

The disulfide-bonded TRH1 peptides from both fetuin and TβRII were tested for cytokine binding activity using surface plasmon resonance. Cyclized TRH1 peptide from TβRII bound to TGF-β1 with higher affinity than to BMP-2, and the cyclized peptide from fetuin bound preferentially to BMP-2 (Fig. 2*C* and Table I). This result reflected the cytokine-binding preference we had observed for full-length fetuin and that expected for TβRII (37). Furthermore, reduced and alkylated TRH1 peptides showed significant loss of cytokine binding activity as did reduced fetuin protein, suggesting that the disulfide loop stabilizes a preferred TRH1 peptide structure for cytokine binding (Fig. 2*D* and Table I). Loop 2, a disulfide-looped peptide of the same length as TRH1 but divergent in sequence, is found in the C-terminal portion of fetuin (Fig. 3*A*). Cyclized loop 2 peptide did not bind to BMP-2 (Table I). The affinity of TGF-β1 for the looped TRH1 peptide of TβRII was only 10-fold less avid than TGF-β1 binding to the full-length extracellular domain of TβRII, which has also been measured by surface plasmon resonance (39). Finally, in a competitive binding experiment, TRH1 peptide from TβRII and native fetuin glycoprotein, at concentrations approximately 100-fold higher than their respective K_D values, were found to block TGF-β1 binding to the recombinant extracellular domain of TβRII (Fig. 3*B*). Taken together, these results suggest that TRH1 is the primary cytokine recognition site in TβRII and fetuin and imply that sequence differences in TRH1 domains may largely dictate cytokine binding specificity.

With the known bone remodelling effects of both fetuin and TGF-β/BMPs (14, 21), as well as their co-localization in developing bone (23, 40), it is possible that fetuin-cytokine interactions play a role in bone differentiation. To examine this possibility, we cultured rat bone marrow stromal cells in the presence of dexamethasone, a treatment that induces osteogenic differentiation leading to deposition of a calcium-containing matrix after 3 weeks of culture (Fig. 5). Consistent with a bone phenotype, the matrix contained abundant collagen fibrils

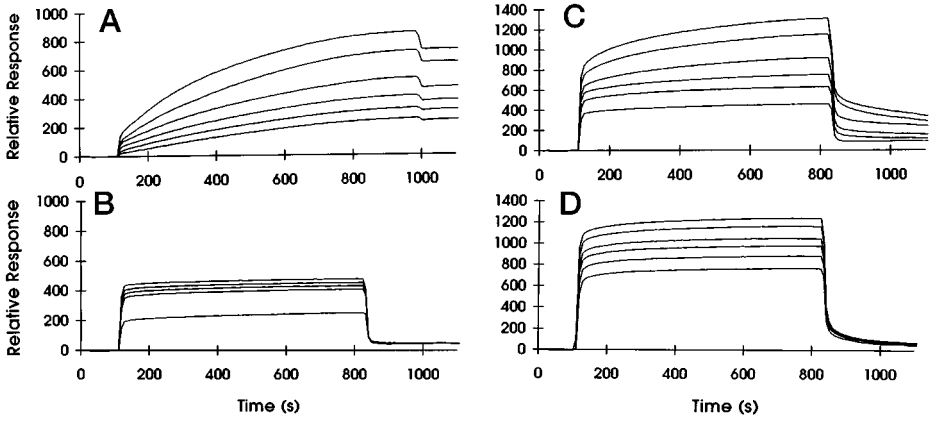

FIG. 2. **Overlay plots of sensograms showing binding of increasing concentrations of fetuin (*A*), BSA (*B*), cyclized (*C*), and reduced (*D*) TRH1 peptide from fetuin to immobilized BMP-2.** Protein in the fluid phase was as follows: 0.21, 0.31, 0.41, 0.62, 0.82, and 1.24 μM (*A*); 0.22, 0.29, 0.44, 0.59, and 0.88 μM (*B*); 87, 130, 174, 217, 283, and 348 μM (*C* and *D*). Relative response for the immobilized cytokine in each experiment was 7450 (*A*) and 5300 (*B*, *C*, and *D*) relative units, respectively. Fetuin did not bind to other immobilized proteins or to blocked dextran surface.

TABLE I
Fetuin and TRH1 binding to TGF-β cytokines

Binding constants for fetuin and TRH1 peptide interactions with TGF-β and BMPs measured using the BIAcore and BIA-evaluation software (Pharmacia) are shown. The change in response with time was plotted for each analyte concentration (*i.e.* d*R*/d*T versus R*), and the slopes of these lines were then plotted as a function of analyte concentration (*i.e.* d(d*R*/d*T*)/d*R versus C*), where the slope yields k_a. The dissociation rate constant, k_d, is obtained after analyte injection is discontinued as the slope of $\ln(R_{t1}/R_{tn})$ *versus* time. K_D values are k_d/k_a. Variance for k_a is standard error of the linear regression plot, and for k_d values are the mean ± range of three or more independent injections. Steady state was observed for fetuin-TGF-β1 binding, and Scatchard plot analysis produced a similar K_D value, 4.6×10^{-6} M. Bracketed numbers are percentage of homology of the cytokines relative to BMP-2. h- and b-fetuin refer to human and bovine forms of the glycoprotein, respectively.

Analyte	Ligand	k_a	k_d	K_D
		$M^{-1}S^{-1}$	S^{-1}	M
b-Fetuin	TGF-β1 (38%)	$8.7 \times 10^2 \pm 2.7 \times 10^2$	$1.9 \times 10^{-3} \pm 0.6 \times 10^{-3}$	2.2×10^{-6}
	TGF-β2 (36%)	$5.5 \times 10^2 \pm 1.2 \times 10^2$	$2.4 \times 10^{-3} \pm 0.2 \times 10^{-3}$	4.4×10^{-6}
	BMP-2 (100%)	$2.4 \times 10^3 \pm 1.2 \times 10^3$	$6.5 \times 10^{-5} \pm 2.1 \times 10^{-5}$	2.7×10^{-8}
	BMP-4 (86%)	$4.2 \times 10^3 \pm 0.2 \times 10^3$	$5.1 \times 10^{-4} \pm 0.1 \times 10^{-4}$	1.2×10^{-7}
	BMP-6 (57%)	$2.9 \times 10^3 \pm 0.5 \times 10^3$	$2.1 \times 10^{-3} \pm 0.3 \times 10^{-3}$	7.1×10^{-7}
h-Fetuin	BMP-2	$2.2 \times 10^4 \pm 0.5 \times 10^4$	$1.1 \times 10^{-3} \pm 0.1 \times 10^{-3}$	5.0×10^{-8}
TRH1 (b-fetuin)	BMP-2	$1.6 \times 10^3 \pm 0.5 \times 10^3$	$3.8 \times 10^{-3} \pm 0.5 \times 10^{-3}$	2.4×10^{-6}
	TGF-β1			$>10^{-5}$
TRH1 (reduced)	BMP-2			$>10^{-5}$
loop2 (b-fetuin)	BMP-2			$>10^{-5}$
TRH1 (TβRII)	BMP-2			$>10^{-5}$
	TGF-β1	$2.2 \times 10^3 \pm 1.3 \times 10^3$	$2.3 \times 10^{-3} \pm 0.5 \times 10^{-3}$	1.0×10^{-6}
TRH1 (reduced)	TGF-β1			$>10^{-5}$

with discrete areas of mineralization as seen at the ultrastructural level, as well as bone sialoprotein, alkaline phosphatase, and osteopontin detected by Western blotting (data not shown).

During the first week of culture, the bone marrow cells proliferate, and TGF-β1 has been shown to inhibit cell proliferation and osteogenesis during this period (41). However, in the latter phases of osteogenic differentiation, TGF-β is necessary for deposition of matrix (21). Dexamethasone increased the fraction of active *versus* latent TGF-β cytokine in bone marrow cultures but had little effect on total TGF-β levels as has previously been reported (42) (Fig. 5*C*). As shown in Fig. 5*A*, the addition of fetuin during the first week stimulated an increase in cell number consistent with its role as a TGF-β antagonist (Fig. 5*A*). When added during the latter 2 weeks of culture, fetuin blocked deposition of calcium-containing matrix with an IC_{50} of approximately 10^{-5} M, only 5-fold greater than the K_D for fetuin-TGF-β binding (Table I). Neutralizing anti-TGF-β antibodies also inhibited deposition of calcium-containing matrix in dexamethasone-treated cultures (Fig. 5*D*), confirming that TGF-β is required to complete the final stages of differentiation. However, this does not exclude the involvement of other TGF-β cytokines such as the BMPs, which may also be present and bind to fetuin in this cell culture model. The suppressive effect of fetuin on deposition of calcium-containing matrix is not likely due to cytotoxicity, as marrow cell proliferation was stimulated by fetuin during the first week of culture. Furthermore, fetuin did not manifest its effect by inhibiting or inducing activation of latent TGF-β (Fig. 5*C*).

DISCUSSION

Fetuin comprises ~45% of the protein in fetal calf serum and was discovered over 50 years ago, yet its function has remained elusive. In this report, we have shown that fetuin binds to the TGF-β cytokines BMP-2, BMP-4, BMP-6, TGF-β1, and TGF-β2, listed here in order of decreasing affinity. The K_D for fetuin binding to TGF-β1 was determined to be 2×10^{-6} M, a physiologically relevant affinity, as fetuin is $\sim 10^{-5}$ M in postnatal bovine serum and $\sim 10^{-4}$ M in fetal serum (43). TRH1, a disulfide-looped 19-amino acid sequence in fetuin, bound directly to BMP-2 and showed similar on-rates as native fetuin, whereas off-rates were 100 times faster for the peptide. This suggests that the TRH1 sequence is the major cytokine binding site and that other portions of the fetuin may stabilize the binding conformation of the TRH1 loop or present additional surfaces for cytokine binding, thereby slowing the off-rate of fetuin when compared with the peptide.

The TRH1 sequence of fetuin was observed to be similar to a cysteine-flanked 18-amino acid sequence in TβRII. The cyclized TRH1 peptide from TβRII blocked binding of TGF-β1 to the extracellular domain of TβRII in an ELISA assay, suggesting that the receptor TRH1 domain also bound cytokine. Furthermore, TRH1 peptide from TβRII bound directly to TGF-β1 in a disulfide-looped form but poorly as a linear peptide, suggesting that the looped peptide assumed a structure similar to TRH1 in the context of full-length TβRII. Although the disulfide bonding arrangement in the extracellular domain of TβRII has not been

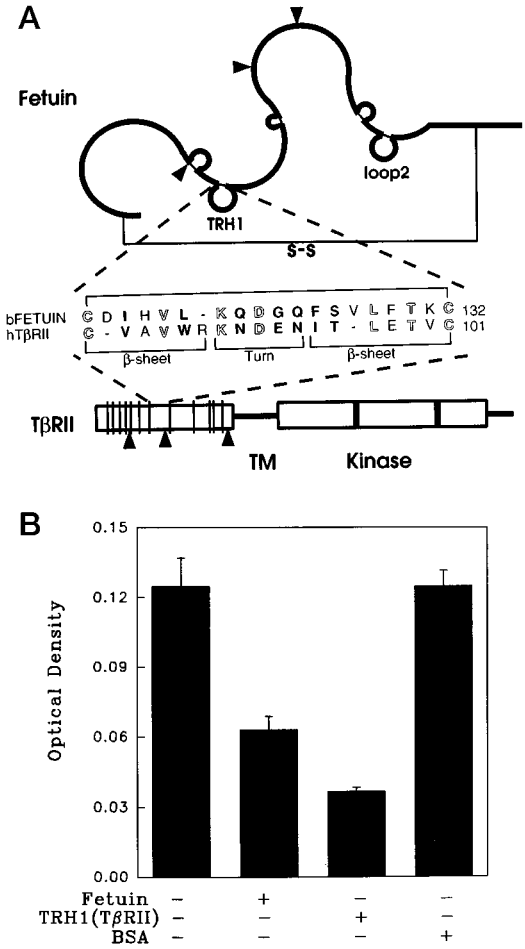

FIG. 3. *A*, schematic of bovine fetuin and human TβRII showing the TGF-β receptor homology 1 (TRH1) domains. The TRH1 domain in fetuin is at position 114–132, and the inactive loop 2 at positions 230–248 as indicated (3). *TM* and *kinase* indicate transmembrane domain and protein kinase domains of TβRII, respectively. The TRH1 domain of TβRII is at positions 84–101 of the open reading frame. The Chou-Fasman (60) and Garnier *et al.* (61) methods of predicting secondary structure produced similar results for the TRH1 domain of fetuin and TβRII as indicated *below* the sequences. *B*, competition of TGF-β1 binding to extracellular domain of TβRII by fetuin and by the TRH1 peptide from TβRII. TβRII was immobilized on plastic ELISA plates, and bound TGF-β1 was detected with secondary antibodies as described under "Experimental Procedures." Determinations were made in triplicate.

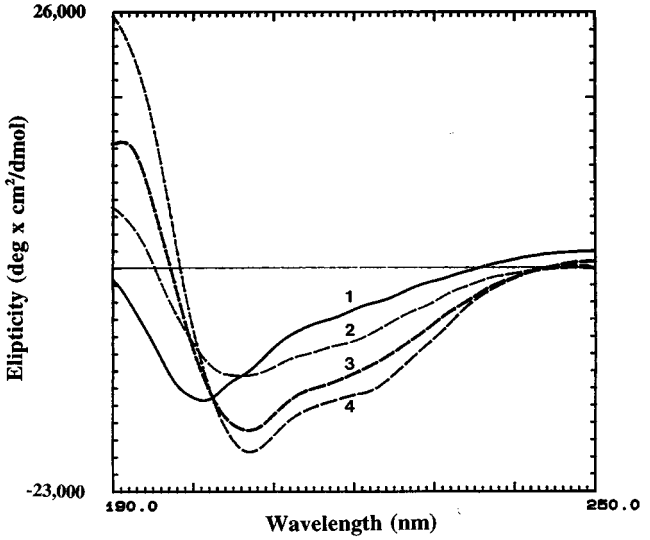

FIG. 4. **Low ultraviolet circular dichroism (CD) spectral analysis of the TRH1 peptide from TβRII in water (*1*) and 25% (*2*), 50% (*3*), and 75% (*4*) 2,2,2 trifluroethanol.**

determined, our results suggest that residues Cys^{114}–Cys^{130} flanking the TβRII domain form a disulfide bridge. The soluble extracellular domain of TβRII (sTβRII) has been reported to bind immobilized TGF-β1 with a K_D of 1×10^{-7} M by surface plasmon resonance measurements (39). This compares favorably with the TRH1 peptide of TβRII, which bound only 10-fold less avidly as measured in this study. However, cells commonly bind and respond to TGF-β in the 1–10 pM range *in vitro*, several orders of magnitude lower than the K_D reported for type II receptor binding. On the other hand, cytokine-receptor cross-linking studies show that TβRII is only one of several components in the TGF-β receptor complex (32). In the absence of TβRI and the presence of only TβRII and betaglycan (*i.e.*, type III receptor), TGF-β binds with an affinity of approximately 8×10^{-10} M (37). Betaglycan has been reported to enhance TGF-β binding to cell surface TβRII by at least 50-fold (44). Therefore, in the absence of both betaglycan and TβRI, TGF-β affinity for cell surface TβRII is expected to be approximately 4×10^{-8} M. Furthermore, TβRII has been shown to exist as a homo-oligomer at the cell surface in the absence of cytokine, which would be expected to enhance binding compared with monomeric TβRII (45, 46). Taken together, these considerations suggest that the surface plasmon resonance measurements provide a reasonable estimate of monomeric TβRII affinity for TGF-β1 at 1×10^{-7} M (39). Therefore, our data indicate that TRH1 is the primary binding domain in TβRII, showing only 10-fold lower affinity as a cyclized peptide compared with the full-length extracellular domain of TβRII. In contrast with these affinity measurements, Lin *et al.* (47) expressed a secreted extracellular domain of TβRII in COS cells and showed binding to ^{125}I-TGF-β1 in solution with 500-fold higher affinity than the surface plasmon resonance measurements (*i.e.* 2×10^{-10} M). However, it is possible that proteoglycans such as soluble betaglycan or other factors in the COS cell supernatants may have affected binding and resulted in an overestimate of affinity.

Fetuin as well as cyclized TRH1 peptide from fetuin bound preferentially to BMP-2, while TRH1 from TβRII bound with greater affinity to TGF-β1, suggesting that for type II receptors and binding proteins with a TRH1 domain, variations in TRH1 sequence may dictate cytokine binding specificity. However, neither fetuin nor cell surface type II receptors show strictly monogamous cytokine binding patterns (32). In this report, fetuin was observed to bind multiple TGF-β cytokines with affinities that vary over 200-fold. Recently, BMP-7/OP-1 has been shown to bind the activin type II receptor on Mv1Lu cells and inhibit cell growth (48). This degeneracy in cytokine binding specificity suggests that "cross-talk" between cytokines, receptors, and antagonists occurs and provides an additional level of complexity to TGF-β cytokine responses.

Soluble binding proteins have been reported for a number of growth factors, peptide hormones, and cytokines. The role of these binding proteins may be to modulate activity, regulate clearance, and influence factor distribution in various tissues. In addition to K_D, a measure of affinity at steady-state, the on- and off-rates provide information on the turnover of protein-protein interactions. For receptor-ligand interactions, the on-rates are often rapid and only limited by diffusion rates with k_a of $<10^{-5}$ M^{-1} s^{-1}, and affinities vary as functions of the off-rates (49). In contrast, the on- and off-rates for fetuin-cytokine binding were observed to be relatively slow, which may be an important feature of cytokine antagonists. Similarly, follistatin-activin binding has an affinity of 10^{-9} M, but the kinetics

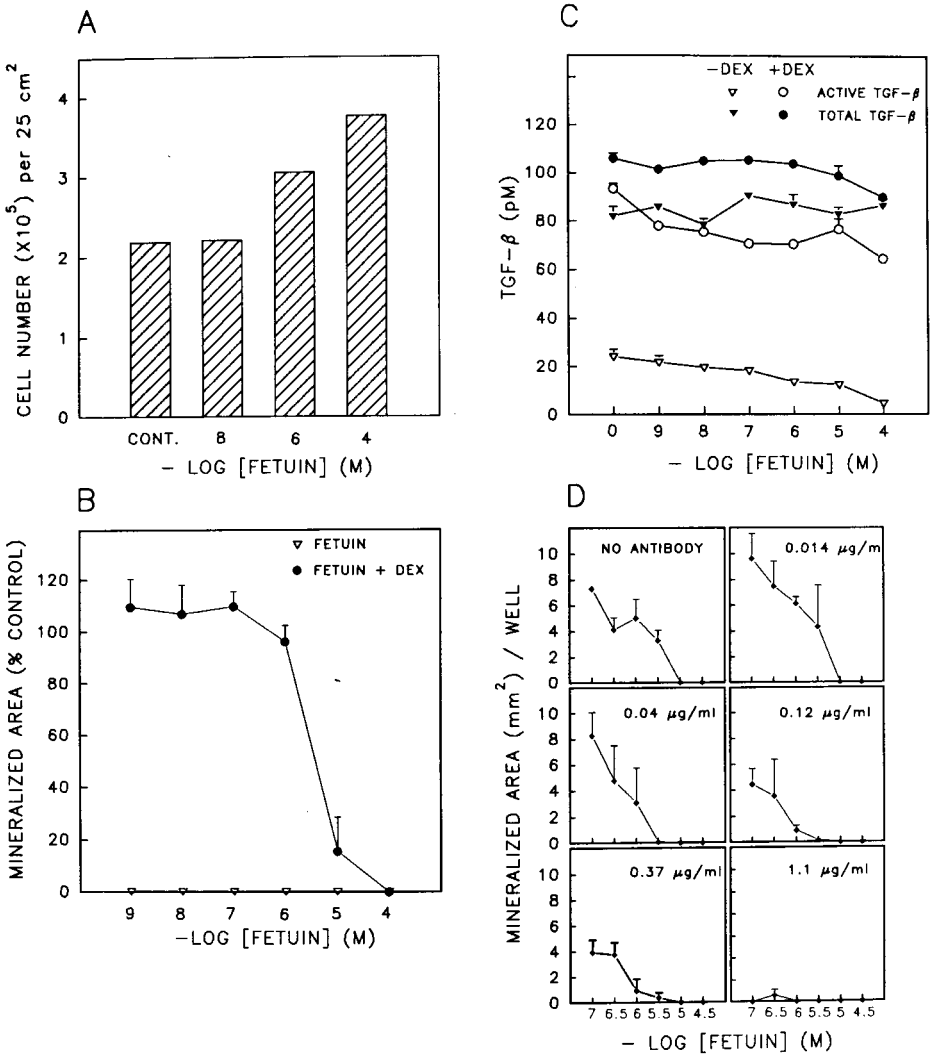

FIG. 5. **Fetuin neutralizes TGF-β and blocks osteogenic differentiation of rat bone marrow cells.** *A*, fetuin stimulates proliferation of rat bone marrow cells treated with dexamethasone (*DEX*) for 6 days. *B*, fetuin inhibits subsequent mineralization in the dexamethasone-treated cultures when present from 1 through 3 weeks, and lacks osteogenic activity alone. Mineralization values were normalized to measurements made in the absence of fetuin, and the results are an average of four experiments. *C*, dexamethasone induces the activation of latent TGF-β in the marrow cultures. Active TGF-β and total TGF-β were measured in dexamethasone-treated and untreated cultures as indicated in the key. *D*, neutralizing anti-TGF-β antibody suppressed mineralization and was additive with fetuin in the 1–3-week culture period.

have been shown to be very slow (50). With the slow binding kinetics and $>10^{-5}$ M concentrations of fetuin in serum and bone, it is possible that fetuin serves as a sink or reservoir for cytokine, which can be released when needed during bone remodelling to stimulate local osteogenesis. BMP-2, TGF-β1, and TGF-β2 also bind to heparin and chondroitin sulfate with slow kinetics,[3] an additional interaction that may limit the diffusion and distribution of cytokines in tissues.

Fetuin as well as neutralizing anti-TGF-β antibodies blocked formation of mineralized bone nodules when added to dexamethasone-treated bone marrow cells in weeks 2 and 3 of culture. The IC_{50} for inhibition of bone nodule formation by fetuin was 10^{-5} M, similar to the fetuin concentration in adult serum (43) and only 5-fold greater than the K_D for fetuin-TGF-β1 binding by surface plasmon resonance. This is consistent with the notion that fetuin acts by antagonizing dexamethasone-induced TGF-β1 activity in the marrow cell cultures. In these experiments, fetuin was present in the 15% fetal calf serum of the culture medium at concentrations of $2–3 \times 10^{-5}$ M, and therefore the IC_{50} value represents only a 30–50% increase in fetuin concentrations. This suggests that a relatively small or incremental change in fetuin concentration can significantly affect TGF-β cytokine responses. Although the IC_{50} value was appropriate for neutralization of TGF-β1 in the dexamethasone-treated bone marrow cell cultures, fetuin bound BMP-2 with higher affinity than TGF-β1 *in vitro*, and therefore it is possible that BMPs and other members of the TGF-β cytokine family were also neutralized in this assay. BMP-2 and -7 have been shown to bind to type I receptors alone; however, the recently cloned BMP-RII appears to contribute to cytokine binding and signaling through ActRI (38). Biological responses to BMPs in tissue culture generally occur at 100-1000-fold higher cytokine concentrations than responses to TGF-β1, which may be due to less efficient use of type II receptors. It is possible that in addition to acting as cytokine antagonist in solution, fetuin might act as a co-receptor at the cell surface and under certain conditions enhance BMP binding to signaling receptors.

A number of observations are consistent with a role for fetuin in bone differentiation and remodelling. During embryogenesis, fetuin mRNA is expressed in the developing mouse limb buds at day 12 (12) when BMP-2 and BMP-4 transcripts are also observed (24, 51). Fetuin, TGF-β, and subsets of the BMP cytokines are expressed at similar times in several other embryonic tissues, such as brain, heart, lung, nervous system, and kidney (12, 51, 52), where their interaction may influence tissue development. In Paget's disease, an affliction of increased bone turnover with disordered and thickened bone, serum fetuin levels are depressed (53), whereas fetuin is elevated in patients afflicted with a common form of mild osteogenesis imperfecta where bone mass is lost (54).

TGF-β has been implicated in the pathogenesis of a number of fibrotic disorders (55). TGF-β is elevated in dermal wounds (56), where it appears to speed wound closure, thus reducing

[3] M. Demetriou, C. Binkert, B. Sukhu, H. C. Tenenbaum, and J. W. Dennis, unpublished data.

the chances of infection but in the process resulting in scar formation. Neutralizing antibody to TGF-β has been shown to significantly reduce scarring of dermal wounds (57) and to suppress intimal hyperplasia in a rat model of artery injury (58). Similarly, in a rat model of glomerulonephritis, neutralizing anti-TGF-β1 antibody as well as the TGF-β-binding proteoglycan decorin blocks matrix accumulation in glomeruli (55, 59). Our findings suggest that fetuin or antagonists based on the TRH1 peptide structure might also be useful in the clinic as TGF-β cytokine antagonists.

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