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Changes with Age of the Rat Fetuin Concentration in Serum and Its mRNA Expression[1]

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Rat fetuin, a counterpart of human α_2-HS glycoprotein and bovine fetuin, shows strong intermolecular binding and association with other serum proteins. Therefore, to measure its concentration in rat serum, we pretreated serum samples with 1% SDS plus 5% (*ca.* 0.7 M) 2-mercaptoethanol at 100°C for 3 min, and then subjected them to SDS-PAGE under reducing conditions followed by Western blotting. We found that the fetuin concentrations in normal rat serum determined by Western blotting were 2.5-4.5 mg/ml. These concentrations were three orders of magnitude higher than the previously reported concentrations. We also tried to measure the fetuin concentration in rat serum by means of an enzyme-linked immunosorbent assay after treatment of the samples with 0.1% sodium dodecyl sulfate (SDS) plus 10 mM 2-mercaptoethylamine at 100°C for 3 min, but it gave a value of about 1/4 of that on Western blotting. Rat fetuin is expressed mainly in the liver, with a peak 2-4 weeks after birth, as determined by Northern blot analysis. The fetuin mRNA level in the liver changes almost in parallel with its serum concentration. The tibia also expresses fetuin, but much less than the liver.

Key words: age, fetuin, mRNA, rat, serum concentration.

Fetuin, a fetal protein found in bovine serum by Pedersen in 1944 (*1*, and for a review, see Ref. *2*), has been shown to be the bovine counterpart of human α_2-HS glycoprotein,[5] a major sialoprotein in plasma discovered and purified independently by two groups in the early 1960s (*3, 4*). Fetuins have been shown to be members of the cystatin superfamily (*5*), and have been proposed to have many biological functions (*2*). For example, fetuins are thought to be factors in the immune system (*6, 7*), promoters of opsonization (*8*), trophic factors or modulators in brain development (*9*), and carriers or modulators of biological active components such as fatty acids (*10*) and growth factors (*11-13*). In addition, since fetuins are known to accumulate in mineralized tissues, *e.g.* bone (*14-17*) and dentin (*18*), they have been suggested to be involved in mineralization or bone remodeling (*19, 20*). The full cDNA sequences of fetuins from various species, including human (*21*), bovine (*22*), sheep (*23*), pig (*23*), mouse (*24*), and rat (*25, 26*), have been reported (for details, see Ref. *2*), and rat fetuin has been shown to be identical with pp63 (*25, 26*), a natural tyrosine kinase inhibitor of the rat insulin receptor (*11*).

In rabbits, plasma fetuin has been shown to originate in the liver by perfusion experiments (*14*). However, in other animals, such as fetal sheep (*27, 28*), many other tissues besides the liver, such as the central nervous system, kidneys, gut, skeletal muscle, and bone, have been shown to express fetuin (for a review, see Ref. *2*). Extensive and systematic studies on the fetuin concentrations in plasma and cerebrospinal fluid have been performed in artiodactylian animals (cattle, sheep, goats, and pigs). In fetuses of these animals, its concentration is high (in fetal cattle and pigs, fetuin accounts for 40-50% of the total plasma protein) but it decreases to less than 1% of the total plasma proteins in adults (0.4-0.5 mg/ml) (*2*). Immunohistochemical studies on the tissue distribution of fetuin in human fetuses, and changes in its concentrations in plasma and cerebrospinal fluid from the fetal to adult stage (*2, 29-31*), and in the tissue distribution of its expression in mice (*24*) have also been reported. However, little is known

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Abbreviations: DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HGF, hepatocyte growth factor; IL, interleukin; PBS, phosphate-buffered saline; SSC, 150 mM NaCl/15 mM sodium citrate (pH 7.0); TPBS, 0.05% Tween 20 in PBS.

[5] Nomenclature for fetuins: According to the suggestion by Haasemann *et al.* (*42*), proteins from various species characterized by extensive sequence similarity with bovine fetuin are denoted as "fetuins" in this paper. For human fetuin, the original designation, "α_2-HS glycoprotein" (*3, 4*), is used synonymously. The rat tyrosine kinase inhibitor of the insulin receptor was previously denoted as "pp63" (*11*).

about fetuin expression or even its serum concentration in rats.

In this paper, we report that the serum concentration of fetuin in rats changes almost in parallel with the fetuin mRNA level in the liver, with a peak 4 weeks after birth, as determined by means of a modified enzyme-linked immunosorbent assay (ELISA). We also report that the serum concentration of fetuin in young and adult rats ranges from 2.5 to 4.5 mg/ml, as determined by Western blotting, these values being much higher than the previously reported concentrations of 2-3 μg/ml (*11*).

MATERIALS AND METHODS

Materials—[α-^{35}S]UTP (46.3 TBq/mmol) and [α-^{32}P]-dCTP (111 TBq/mmol) were purchased from Du Pont NEN Life Science Products, Boston, MA; Denhardt's solution was from Amresco, Solon, OH; dithiothreitol (DTT) and ribonucleotides (rCTP, rATP, and rGTP) were from Promega, Madison, WI; horseradish peroxidase (grade I), T3 and T7 DNA-dependent RNA polymerases, transcription 10×buffer, RNase inhibitor, RNase-free DNase I, RNase A, yeast tRNA, *Sma*I, and *Pst*I were from Boehringer Mannheim GmbH, Mannheim, Germany; *Eco*RI was from MBI Fermentas, Buffalo, NY; paraformaldehyde (extra pure) was from Merck, Darmstadt, Germany; deionized formamide, diethyl pyrocarbonate (DEPC), and acetic anhydride were from Nacalai Tesque, Kyoto; 2-mercaptoethylamine hydrochloride and salmon testes DNA were from Sigma, St Louis, MO; dextran sulfate (mean mol. wt., 5,000), triethanolamine, and Ultrogel AcA 34 were from Wako Pure Chemicals, Osaka; biotinylated anti-mouse IgG (H+L) (affinity pure) and a Vectastain ABC kit were from Vector Laboratories, Burlingame, CA; silanized slides were from Dako, Tokyo; microtiter plates with 96-well (Sepalate 8F, Stripwell-H type) were from Sumitomo Bakelite, Tokyo; and Bio-Max MR X-ray film and developer were from Eastman Kodak, Rochester, NY. Rabbit anti-(rat fetuin) polyclonal antibodies were prepared and affinity purified as described previously (*26*). Mouse anti-(rat fetuin) monoclonal antibody (6-C) was prepared as described previously (*16*). The two antibodies were specific for rat fetuin, did not cross-react with mouse fetuin, and gave single bands at 59 kDa corresponding to rat fetuin when rat serum was analyzed by Western blotting (*16, 26*). Rat fetuin was purified from rat bone matrix as described previously (*16*). Other materials used for SDS-PAGE, electrophoresis, ELISA, and Northern and Western blotting were as described (*13, 16, 26, 32*).

Determination of Rat Fetuin in Serum by Western Blotting—Serum samples from rats of 2, 14, 28, 42, and 77 days old were diluted 1:1,000 with the sample buffer for SDS-PAGE (*33*) containing 1% SDS and 5% 2-mercaptoethanol, and aliquots (10 μl) were subjected to SDS-PAGE. SDS-PAGE was performed essentially as described by Laemmli (*33*) in a 4-20% linear gradient separating gel (Multi Gel; Daiichi Pure Chemicals, Tokyo) under reducing conditions. The running conditions for SDS-PAGE and Western blotting were performed as described previously (*34*). Briefly, after SDS-PAGE, proteins were transferred electrophoretically to a nitrocellulose membrane (Nippon Bio-Rad) in 20 mM Tris/150 mM glycine buffer (pH 8.0) containing 20% methanol overnight at 0.09 A. The membrane was blocked with 4% non-fat dry milk (Snow Brand, Tokyo) in PBS (blocking buffer) for 1 h at 37°C, washed 3 times with 0.05% Tween 20 in PBS (TPBS) for 5 min each time, and then incubated with affinity purified anti-(rat fetuin) polyclonal antibodies (0.27 μg/ml in blocking buffer) for 1 h at 37°C. The membrane was washed 3 times with TPBS, incubated with 1.55 μg/ml peroxidase-conjugated goat anti-(rabbit IgG) antibodies [affinity pure F(ab'); Jackson Immuno. Res. Lab., West Grove, PA] in blocking buffer at 37°C for 1 h, and then washed 3 times in TPBS. Then the antibodies specifically bound to rat fetuin were visualized by adding 10 ml of 0.5 mg/ml 4-chloro-1-naphthol and 0.015% hydrogen peroxide in PBS containing 16.7% ethanol for 5-10 min. The reaction was stopped by washing with H_2O, and then the intensities of bands were measured using the computer base image analysis system of the NIH Image version 1.51 non-FPU program (Research Service Branch, National Institute of Mental Health, NIH, Bethesda, MD). The concentrations of fetuin in serum were calculated by comparison with those of purified rat fetuin from bone matrix.

Determination of Rat Fetuin in Serum by ELISA—The ELISA for rat serum fetuin we used in this study was a sandwich method consisting of three steps (*35*). Purified rat fetuin was used as a standard. Microtiter plates were coated with 100 μl of mouse anti-(rat fetuin) monoclonal antibody at a concentration of 2 μg/ml in PBS, incubated at 37°C for 1 h, and then washed 5 times with TPBS. A solution of 200 μl of 1% gelatin in PBS was added and after incubation at 37°C for 1 h for blocking, the wells were washed with TPBS as above. Aliquots of 100 μl of serum (diluted to 1:500) or standard rat fetuin (5 μg/ml) serially diluted from 1:1 to 1:16 with blocking buffer containing 0.1% SDS with or without 10 mM 2-mercaptoethylamine were heated for 3 min at 100°C, cooled to room temperature, dispensed into the wells, and then incubated at 37°C for 1 h. Serum and standard samples without treatment with SDS, 2-mercaptoethylamine, and heating were also used as controls. After incubation, the wells were washed with TPBS as above, and 100 μl of affinity purified anti-(rat fetuin) polyclonal antibodies at a concentration of 0.108 μg/ml diluted with blocking buffer containing 1% normal mouse serum was added, followed by incubation at 37°C for 1 h. The anti-(rat fetuin) polyclonal and monoclonal antibodies used in this study do not cross-react with mouse fetuin (Ohnishi *et al.*, unpublished data). After washing with TPBS as above, 100 μl of 0.78 μg/ml peroxidase-conjugated goat anti-rabbit IgG (affinity pure) in blocking buffer was added. After incubation at 37°C for 1 h, the wells were washed with TPBS as above, and then incubated with 100 μl of a solution comprising 0.5 mg/ml *o*-phenylenediamine and 0.015% hydrogen peroxide in 0.1 M citric acid/Na_2HPO_4 buffer (pH 5.0) for 10 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 N H_2SO_4. The peroxidase activity was then determined from the absorbance of wells with a two wavelength microplate reader (model MPR-4i; Tosoh, Tokyo) at 492 nm, with a reference wavelength of 630 nm.

To determine whether or not SDS in samples affects the attachment of mouse IgG to plastic plates, 96-well microtiter plates were coated with 100 μl of anti-(rat fetuin) monoclonal antibody at a concentration of 2 μg/ml in PBS, incubated at room temperature for 1 h, and then washed 5

times with TPBS. After incubation with 200 μl of 4% skimmed milk in PBS (blocking buffer) at 37°C for 1 h for blocking, they were washed with TPBS as above. Then they were incubated with 100 μl of SDS at various concentrations (0 to 1%), dilution being performed with blocking buffer, at 37°C for 1 h, washed with TPBS as above, and then treated with 100 μl of biotinylated anti-mouse IgG (H+L) (affinity pure) diluted 1:100,000 in blocking buffer at 37°C for 1 h. The wells were next washed with TPBS as above, and the antibodies attached to the plastic plates were quantified using a Vectastain ABC kit for mouse IgG. Color development and measurement of absorbance were performed as in the ELISA procedure described above.

Determination of Rat Fetuin in Serum by Means of a Competitive Enzyme Immunoassay Using Rat Fetuin-Peroxidase Conjugate—Rat fetuin was conjugated to horseradish peroxidase through the reaction of maleimide groups introduced into peroxidase and thiol groups generated in fetuin, essentially as described Ishikawa *et al.* (*36*). Briefly, maleimide groups were introduced into 10 mg peroxidase in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) and 2.5 mM *N*-(6-maleimidocaproyloxy)succinimide, and thiol groups were generated in 2 mg of rat fetuin in 1 ml of 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA by reduction with 10 mM 2-mercaptoethylamine. Then the maleimide-peroxidase was allowed to react with thiol groups of the reduced rat fetuin. To remove free peroxidase and free fetuin, we filtered the conjugate through a column of Ultrogel AcA 34 (16×450 mm). The average number of peroxidase molecules conjugated per rat fetuin molecule was 1.0, as determined from the absorbance at 403 and 280 nm (*36*).

For competitive enzyme immunoassaying of rat fetuin, 96-well microtiter plates were coated with 100 μl of affinity purified anti-(rat fetuin) polyclonal antibodies at a concentration of 150 ng/ml in PBS, incubated at room temperature for 1 h, and then washed 5 times with TPBS. For blocking, 200 μl of 1% gelatin in PBS (blocking buffer) was added per well. After incubation at 37°C for 1 h, the wells were washed with TPBS as above. Aliquots (80 μl) of serum samples serially diluted from 1:100 to 1:6,400, and standard rat fetuin (8 μg/ml) serially diluted from 1:1 to 1:32 with blocking buffer containing 0.1% SDS and 10 mM 2-mercaptoethylamine were heated for 3 min at 100°C, cooled to room temperature, and then mixed with 20 μl of 20 μg/ml rat fetuin-peroxidase conjugate. Then the mixtures (total 100 μl) were dispensed into wells and incubated at 37°C for 1 h. The wells were then washed with TPBS as above, and 100 μl of a solution comprising 0.5 mg/ml *o*-phenylenediamine and 0.015% hydrogen peroxide in 0.1 M citric acid/Na_2HPO_4 buffer (pH 5.0) was added. After incubation for 10 min at room temperature, the reaction was terminated by the addition of 100 μl of 1 N H_2SO_4. The peroxidase activity specifically bound to anti-(rat fetuin) antibodies was then determined from the absorbance of wells with a two wavelength microplate reader as described above.

Preparation of a Riboprobe for In-Situ Hybridization—The 369 bp *Pst*I/*Pst*I rat fetuin cDNA fragment encoding nucleotide positions 463-832 (*26*) was subcloned into an expression vector, pBluescript II SK⁻ (Stratagene, La Jolla, CA), using *Pst*I restriction sites. The correct insertion of rat fetuin cDNA into the subcloned vector was confirmed by DNA sequencing with an automated DNA sequencer (Model SQ-5500; Hitachi, Tokyo) according to the instructions for a Delta Taq fluorescent dye-primer cycle sequencing kit (RPN 2336; Amersham Life Science, Buckinghamshire, England). Then large scale preparation of rat fetuin cDNA insert-containing plasmids was performed after transfection into *Escherichia coli*. Plasmids were extracted and purified as described by Sambrook *et al.* (*37*). To generate antisense and sense RNA probes, the plasmids were linearized by incubation with *Sma*I and *Eco*RI, respectively. Linearization of the plasmids was confirmed by the electrophoretic mobility, in a 1.2% agarose gel buffered with 45 mM Tris-borate containing 1 mM EDTA (pH 8.3), of bands stained with ethidium bromide. Antisense and sense cRNA probes were transcribed using T7 RNA polymerase and T3 RNA polymerase, respectively. *In vitro* transcription was performed in a 20 μl reaction mixture as described by Wilcox (*38*) with minor modification. Briefly, the transcription reaction mixture consisted of 1 μl of 1 μg/μl linearized DNA, 2 μl of 10×transcription buffer (Boehringer), 2 μl of 0.1 mM DTT, 1 μl each of 10 mM rATP, rGTP, and rCTP, 8 μl (3.7 MBq) of [α^{35}S]UTP (46.3 TBq/mmol), 1 μl (40 U) of RNase inhibitor, 1 μl of either T3 or T7 RNA polymerase (20 U/μl), and 2 μl of H_2O treated with 1 ml/liter DEPC. The reaction was performed at 37°C for 2 h and stopped by degrading the plasmids with 1 μl of RNase-free DNase I (10 U/μl) for 15 min at 37°C. Protein and free nucleotides were removed, extracted, precipitated, washed, and dried as described by Wilcox (*38*), and then resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA.

In-Situ Hybridization—Time-mated pregnant Wistar/ST rats (Kuroda Experimental Animals, Kumamoto) were killed by cervical dislocation, and 18-day fetuses were collected surgically and snap frozen in powdered dry ice. *In-situ* hybridization was performed as described by Carrasco and Bravo (*39*) with a minor modification. Briefly, 10 μm frozen sections (at −18°C) of 18-day fetuses were placed on silanized slides, air dried for 1 h at room temperature, and then fixed for 1 h at 4°C with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) (PBS). After washing with PBS twice for 5 min each, the slides were immersed in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min. Then they were washed with PBS, post-fixed in 4% paraformaldehyde in PBS at 4°C for 3 min, rinsed with PBS and 0.85% NaCl for 5 min each, and dehydrated by dipping for 2 min in each of an ethanol series, and then twice in 100% ethanol. They were then air-dried and hybridized with riboprobes overnight at 50°C under parafilm coverslips in a humidified chamber (the internal wall being covered with tissue paper soaked in 50% formamide/4×SSC, 1×SSC consisting of 150 mM NaCl and 15 mM sodium citrate, pH 7.0). The hybridization buffer used consisted of 50% deionized formamide, 1×Denhardt's solution, 10% dextran sulfate, 5 mM EDTA, 20 mM Tris-HCl (pH 7.4), 10 mM DTT, 250 μg/ml denatured salmon sperm DNA, and 0.3×10^9 cpm/ml of either antisense or sense cRNA probe (denatured by heating at 80°C for 5 min and then chilled on ice). After hybridization, the slides were washed in 5×SSC containing 10 mM DTT prewarmed at 50°C for 30 min with constant agitation, and then the coverslips were allowed to slide off. The sections were further washed in 2×SSC containing 10

mM DTT and 50% formamide for 30 min at 65°C, rinsed a few times in 1×RNase buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.5 M NaCl, pH 8.0), and then incubated with RNase A (20 μg/ml) in RNase buffer for 30 min at 37°C. Then they were rinsed in RNase buffer at 37°C for 5 min, in 2×SSC at 37°C for 30 min, and in 0.1×SSC at 65°C for 30 min, and then dehydrated in a graded ethanol series, air-dried and exposed to Bio Max MR X-ray film overnight at −80°C.

Northern Blotting—For Northern blot analysis, the livers and tibia of Wistar/ST rats of 2, 14, 28, 42, and 77 days old were removed surgically as quickly as possible and frozen in liquid nitrogen. Isolation of total RNA and Northern blotting were performed as reported previously (*26*). Briefly, total RNA was extracted from the livers and tibia of rats of 2, 14, 28, 42, and 77 days old with guanidium thiocyanate and then centrifuged in a cesium chloride solution as described by Sambrook *et al.* (*37*). [α-^{32}P]-dCTP labeled cDNA, 6.3×10^6 cpm/ml, was used as a probe. The blots were exposed to either Bio Max MR X-ray film at −80°C or a Bioimaging plate (Fujifilm, Tokyo). The X-ray film was developed according to the manufacturer's protocol. Equal loading of lanes was checked by ethidium bromide staining (*37*).

Statistical Evaluation—Statistical evaluation of differences in the mean values of the groups was preformed by means of Student's *t* test for unpaired samples.

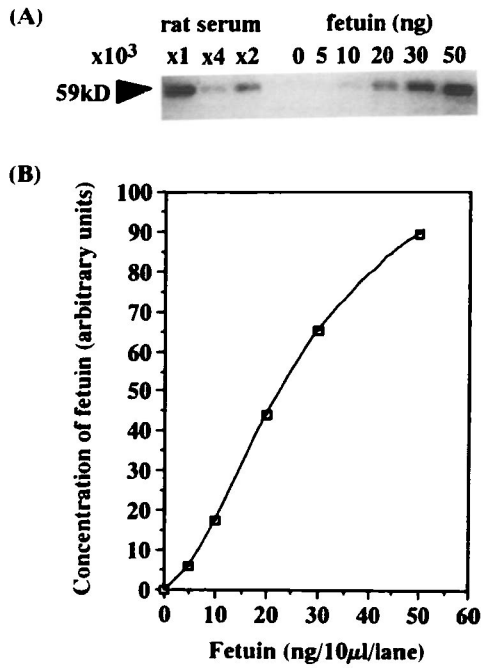

Fig. 1. **Standard curve for rat fetuin on Western blotting.** Panel A: Serum from a 4-week-old rat and rat fetuin purified from bones were diluted as indicated in a loading buffer containing 1% SDS and 5% 2-mercaptoethanol, and then boiled for 3 min. Then aliquots (10 μl) were subjected to SDS-PAGE (4-20% linear gradient) under reducing conditions, followed by Western blotting as described under "MATERIALS AND METHODS." The factors of dilution of serum and the amounts of standard rat fetuin (ng/lane) are indicated above, and the position of rat fetuin (59 kDa) is indicated on the left. Panel B: Intensities of the immunoblotted bands in (A) determined by densitometric quantitation. Values are plotted as a standard curve.

RESULTS

Serum Fetuin Concentrations in Rats of Different Ages—As we reported previously (*16*), rat fetuin in serum was tightly bound to serum albumin and some other protein(s), and the complex(es) could only be split by reverse phase high-performance liquid chromatography after treatment of the samples with 1% SDS and 5% 2-mercaptoethanol at 95°C. Therefore, we thought that determination of serum fetuin by ELISA without pretreatment of samples may result in underestimation of its concentration. To confirm this, we first determined the concentrations by ELISA with or without pretreatment of the samples, and found that the serum fetuin concentrations in a rat of 4 weeks old were 5.46, 82.0, and 988 μg/ml serum in samples without pretreatment, with pretreatment with 0.1% SDS only at 100°C for 3 min, and with pretreatment with 0.1% SDS plus 10 mM 2-mercaptoethylamine at 100°C for 3 min, respectively. These results showed that determination of rat fetuin in serum resulted in erroneously low values of at

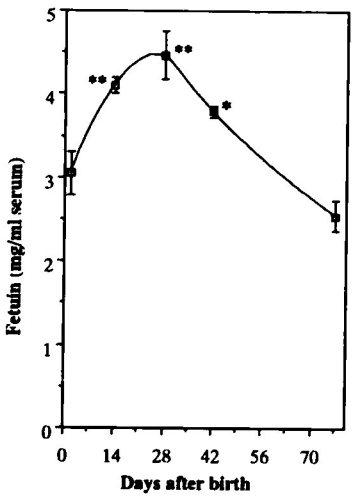

Fig. 2. **Change with age of the rat fetuin concentration in serum determined by Western blotting.** Sera (1:1,000 dilution) of rats of different ages were analyzed by Western blotting as described in the legend to Fig. 1. Points and bars show means and SDs of the values for 3 rats. $^*p<0.01$ and $^{**}p<0.005$ *vs.* values at 2 days old.

TABLE I. **Serum concentrations of fetuin in rats of different ages determined by Western blotting and ELISA.** Sera (1:1,000 dilution) of rats of different ages were analyzed by Western blotting as described in the legend to Fig. 1. The same sera as used for Western blotting were diluted 1:500, and then pretreated with 0.1% SDS and 10 mM 2-mercaptoethylamine at 100°C for 3 min, as described under "MATERIALS AND METHODS," and then fetuin was measured by ELISA. Data are expressed as means±SDs for 3 rats, and the figures in parentheses are % of that of 2-day-old rats. The ratio was calculated from the data for the same rat.

Age of rats (days)	Serum fetuin concentration (mg/ml)		Ratio (Western/ELISA)
	Western blotting	ELISA	
2	3.05±0.26	0.467±0.027	6.53±0.44
14	4.10±0.10[b] (134)	1.009±0.099[a] (215)	3.98±0.38
28	4.46±0.29[b] (146)	1.101±0.031[a] (235)	4.12±0.22
42	3.79±0.07[c] (124)	0.928±0.106[b] (198)	4.01±0.42
77	2.54±0.18 (83)	0.630±0.023[b] (135)	4.21±0.17

[a]$p<0.001$, [b]$p<0.005$, and [c]$p<0.01$ *vs.* 2-day-old rats.

least 1/200 when the determination was performed without pretreatment of the samples. These results also suggested that pretreatment of serum samples with higher concentrations of a sulfhydryl reagent may be necessary to obtain accurate values for the fetuin concentration in rat serum. However, a high concentration of a sulfhydryl reagent (*e.g.* 5% 2-mercaptoethanol) reduces the biological activity of antibodies for ELISA (*36*).

To use a higher concentration of a sulfhydryl reagent for the pretreatment of samples, we next tried to measure the rat fetuin concentration by SDS-PAGE under reducing conditions followed by Western blotting. Serum samples were pretreated with 1% (w/v) SDS plus 5% (v/v) 2-mercaptoethanol, and boiling for 3 min. Figure 1 shows the standard curve for rat fetuin obtained on Western blotting. Using this standard curve, the concentrations of fetuin in serum from a rat of 4 weeks old at different dilutions were almost the same, namely, 4.8, 4.8, and 5.2 mg/ml serum at 1:1,000, 1:2,000, and 1:4,000 dilution, respectively. This value (mean = 4.9 mg/ml) was about 5 times that obtained on the ELISA described above. Therefore, we determined the concentrations of fetuin in rat serum at different ages by Western blotting and found that the serum fetuin concentration in rats ranged from 2.5 to 4.5 mg/ml with age (Fig. 2 and Table I). The concentration reached a peak about 14 to 28 days after birth and the peak concentration was about 1.5 times that at 2 days after birth.

To compare the data obtained on Western blotting and ELISA, we re-assayed the samples used for Western blotting by ELISA. Before subjecting the serum samples to ELISA, they were pretreated with 0.1% SDS plus 10 mM 2-mercaptoethylamine at 100°C for 3 min (Table I). Except for rats of 2 days old, the ratio of the concentrations of rat fetuin in serum determined by Western blotting and ELISA was about 4, indicating that determination of serum fetuin concentration by ELISA gives erroneously low values, even when the samples are pretreated as described above.

We also tried to determine the serum fetuin concentration in a rat of 4 weeks old by means of a competitive immunoassay using peroxidase-conjugated rat fetuin. The concentration was calculated to be 2.66 mg/ml in a sample pretreated with 0.1% SDS and 10 mM 2-mercaptoethylamine. This value was about 2.5 times that determined on ELISA but about half that on Western blotting, and the peroxidase activities in the wells were very weak and the data for different samples showed great variation. The intra-assay coefficient of variation (CV) was more than 10%. Therefore, we could not obtain accurate values for the concentration of fetuin in serum with this assay system.

Expression of Fetuin in Rats—Because the change in the rat fetuin serum concentration with age reported above may be the result of a change in its mRNA expression, we next measured the fetuin mRNA levels in rats of different ages. To determine which tissue(s) expresses fetuin mRNA in rats, we first performed *in situ* hybridization of whole rat bodies. Figure 3 shows autoradiographs of longitudinal sections of a 18-day rat fetus hybridized with ^{35}S-riboprobes. Strong hybridization signals for fetuin mRNA were detectable in the liver but not in other tissues, including bones such as the calvarium under the conditions used.

Next we examined the change in the level of fetuin mRNA expression in rat liver in relation to development after birth by Northern blotting. As shown in Fig. 4, fetuin mRNA expression increased after birth to reach a peak 14 to 28 days after birth and then decreased. The mRNA level in 77-day-old rats was about half the peak value and lower than that in 2-day rats (Fig. 4C). Similar results were obtained in two other series of experiments. These data are consistent with those for the serum concentration of fetuin described above.

As we reported previously, rat bone expresses fetuin mRNA (*26*), and osteoblast-rich cells in primary culture prepared from newborn rat calvaria produce and secrete

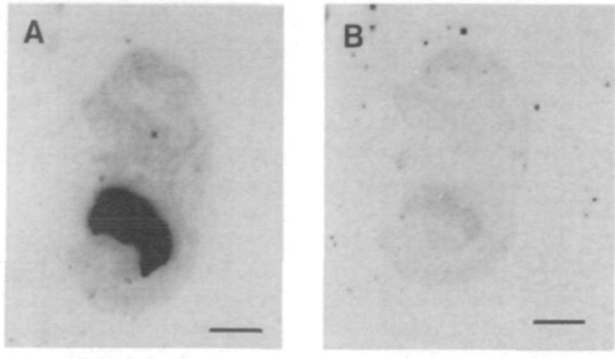

Fig. 3. **Fetuin gene expression in the rat.** Longitudinal sections of the whole body of a 18-day-old rat fetus were processed for *in-situ* hybridization as described under "MATERIALS AND METHODS." Serial sections were probed with a [^{35}S]UTP-labeled antisense (A) or sense (B) riboprobe transcribed from linearized cDNA for rat fetuin. Note that positive expression is only observed in the liver in (A). Bars, 5 mm.

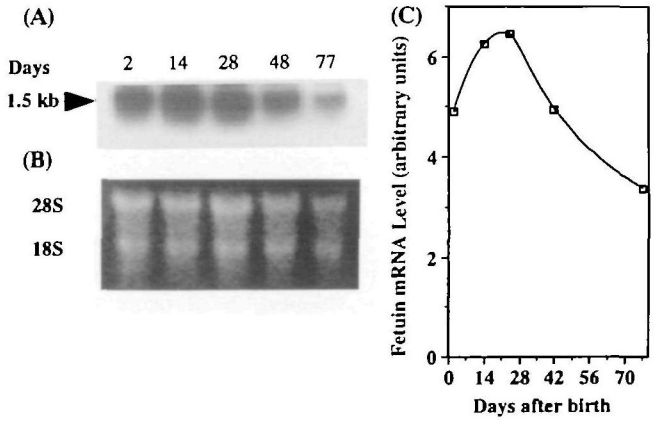

Fig. 4. **Change with age in the expression of rat fetuin mRNA in the liver.** Panel A: Total RNAs (5 μg/lane) extracted from the livers of rats of different ages were electrophoresed and transferred to a nylon membrane, and then the membrane was hybridized with ^{32}P-labeled cDNA for rat fetuin as described under "MATERIALS AND METHODS." The blots were visualized with a Bioimaging analyzer. The position of rat fetuin mRNA (1.5 kb) is indicated on the left, and the ages of the rats are indicated at the top in days after birth. Panel B: Ethidium bromide staining of the agarose gel used for the Northern blotting in (A). The positions of ribosomal RNAs are indicated on the left. Panel C: For determination of the mRNA level, the intensities of the bands of mRNA in (A) were measured with a densitometer, and the relative mRNA values are plotted relative to those for 28S ribosomal RNA in (B).

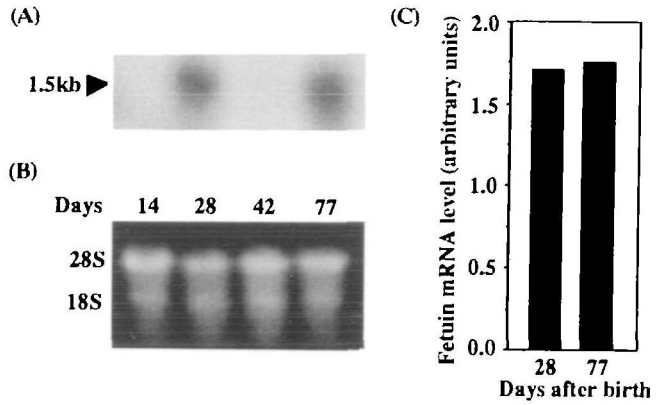

Fig. 5. **Expression of rat fetuin mRNA in the tibia.** Panel A: Northern blot analysis of total RNA (15 μg/lane) extracted from the tibiae of rats of the indicated ages (top) and analyzed as described in the legend to Fig. 4, except that the blots were visualized by autoradiography. The position of rat fetuin mRNA (1.5 kb) is indicated on the left. Panel B: Ethidium bromide staining of the agarose gel used for the Northern blotting in (A). The positions of ribosomal RNAs are indicated on the left. Panel C: For determination of the mRNA level, the intensities of the bands of mRNA in (A) were measured and normalized as described in the legend to Fig. 4. Relative mRNA values are shown.

immunoreactive fetuin (*16*). Therefore, we examined the levels of fetuin mRNA in the tibiae of rats of different ages. Although the levels of fetuin mRNA in the tibia were lower than those in the liver, the mRNA was detectable in rats of 28 and 77 days old, but not in those of 2, 14, and 42 days old (Fig. 5, the datum for a rat of 2 days old is not shown). The reason for the transient repression of fetuin expression in the tibia is not clear, but we obtained similar results in two other series of experiments.

DISCUSSION

Rat fetuin synthesized and secreted by the liver has been shown to be phosphorylated, but to be dephosphorylated during circulation (*11, 26, 32*). Therefore, in rat serum, fetuin should be a mixture of phosphorylated and dephosphorylated forms. However, the anti-rat fetuin antibodies we used in this study recognize both forms of fetuin (*26, 32*). Thus, the serum fetuin values reported in this paper should be for "total fetuin." We have not yet been able to raise an antibody which specifically recognizes either phosphorylated or dephosphorylated rat fetuin.

During our previous studies on fetuin (*16, 26*), we noticed that rat fetuin exhibits tight intermolecular binding and strong binding to other proteins such as albumin, and that the resulting complexes could be split by treatment with either 0.6 N HCl for 2 days or 1% SDS plus 5% (v/v) (*ca.* 0.70 M) 2-mercaptoethanol at 95°C for 3 min. Therefore, we suspected that measurement of fetuin in plasma or sera by means of an ELISA without pretreatment of the samples might give erroneously low values. However, SDS may detach the mouse monoclonal antibody from the coated plastic dishes. Therefore, we first examined whether or not SDS affects the attachment of mouse IgG to plastic dishes, and found that even 1% SDS did not affect its attachment (data not shown). We also examined the concentration of SDS sufficient for the pretreatment of serum samples and obtained almost similar values with 0.1-1.0% SDS (data not shown). Another problem with the enzyme-immunoassay is the sulfhydryl reagent, because it reduces the disulfide bond(s) in the antibodies and may influence their biological activity. In this connection, Ishikawa *et al.* (*36*) reported that 10 mM 2-mercaptoethylamine does not influence the specific binding of antibodies to an antigen. Therefore, we pretreated serum samples with 0.1% SDS plus 10 mM 2-mercaptoethylamine at 100°C for 3 min and then subjected them to ELISA with the sandwich method. In this way, we found that the levels were about 200 times those without pretreatment.

To confirm the results obtained on ELISA, we measured rat fetuin in serum by means of a competitive immunoassay using peroxidase-conjugated rat fetuin and polyclonal antibodies to rat fetuin. When a serum sample of a 4 weeks old rat was pretreated in the same way as for the ELISA described above, the serum concentration of fetuin was calculated to be 2.66 mg/ml, which was about 2.5 times that obtained on ELISA (0.99 mg/ml). This discrepancy may be due to the difference in the necessary epitope number of the ligand in the two assays. Since two anti-(rat fetuin) antibodies are needed for a sandwich ELISA, the ligand must have at least two exposed epitopes during the assay. However, for competitive immunoassay, the ligand requires only one exposed epitope for polyclonal anti-(rat fetuin) antibodies. This discrepancy also suggests that pretreatment of serum samples with 0.1% SDS plus 10 mM 2-mercaptoethylamine was not sufficient to split the rat fetuin from other plasma proteins or its intermolecular linkages. Therefore, we tried to determine the fetuin concentration in rat serum by SDS-PAGE under reducing conditions followed by Western blotting (Fig. 1). The results showed that the fetuin concentrations were 4-6-fold higher than those obtained on ELISA (Table I). When rat serum was pretreated with 0.1% SDS plus 10 mM mercaptoethylamine at 100°C for 3 min and then subjected to SDS-PAGE followed by Western blotting, the intensity of the band was 27% of that in the case of pretreatment with 1% SDS plus 5% 2-mercaptoethanol at 100°C for 3 min. This indicates that a higher concentration of a sulfhydryl reagent than 10 mM is needed to split fetuin in serum completely.

The plasma concentration of fetuin varies in different species and with age. Fetal plasma of animals of the order, *Artiodactyla*, such as cattle, sheep, goats, and pigs, contains high concentrations of fetuin of about 5 mg/ml, the concentrations reaching more than 10 mg/ml in cattle and goats, but the levels decrease to 0.4-0.5 mg/ml in adults, as determined on quantitative complement fixation assay (*2*). The plasma fetuin concentrations were reported to be 0.31-0.47 mg, 1.1 mg, 0.75 mg, and 0.4-0.6 mg/ml in fetal (*30, 31*), newborn (*31*), young (7-20 years old) (*29*), and adult (*29*) humans, respectively (for details, see Ref. *2*). These concentrations for human plasma fetuin were determined by either radial immunodiffusion (*30, 31*) or immunodiffusion (*29*). There have been no reports on the exact fetuin concentration in the plasma of either mice or rats, although Auberger *et al.* (*11*) estimated that the plasma concentration of fetuin in adult rats was 2-3 mg/liter, based on data on purification of rat fetuin (pp63). In this connection, it is noteworthy that Rauth *et al.* (*25*) reported that the plasma concentration of fetuin in normal adult rats determined by

radioimmunoassay was at least two orders of magnitude higher than the values reported by Auberger *et al.* (*11*), although the authors did not show the actual data.

Fetuin is expressed in various organs, but the rate of its expression varies in different species and with age (for a review, see Ref. *2*). For example, the expression is high in the liver, central nervous system, kidneys, heart, gut, skeletal muscle, lungs, and bone in fetal sheep, but it rapidly decreases to near zero after birth, and the relative fetuin mRNA level in adult sheep liver is 0.1% of that in 60-day fetal liver (*27, 28*). Although no systematic, detailed studies on fetuin expression in other species have been performed, Yang *et al.* (*24*) reported that the level of fetuin mRNA in mouse liver is low at 12 days gestation (only 0.5% of the adult level), but increases during the later part of gestation to a peak 1 to 3 months after birth and subsequently decreases to 50% of the peak level. In this study, we demonstrated that the pattern of the fetuin mRNA level in rat liver was similar to that in mice, but that the peak was observed 2 to 4 weeks after birth (Fig. 4). The pattern of the change in the fetuin concentration in rat serum (Fig. 2) was very similar to that of the mRNA (Fig. 4), suggesting that the liver is the main source of serum fetuin in rats after birth.

In this study, we found that the fetuin concentration in rat serum was 2.5-4.5 mg/ml (Fig. 2 and Table I). Assuming that the total protein concentration in adult rat plasma is 60 mg/ml (*40*), the fetuin concentration in total plasma proteins is thus 7.5% at its peak (1 month old) and 4.2% at 11 weeks old. Thus, fetuin should be the third major plasma protein in adult rats after albumin and globulins. This finding suggests that fetuin may play an important physiological role(s) in adult rats.

Fetuin is now known to be a natural modulator of biological active peptides (for a review, see Ref. *2*). Auberger *et al.* (*11*) showed that phosphorylated rat fetuin antagonized the growth-promoting activity of insulin in FaO cells, and Demetriou *et al.* (*12*) showed that bovine and human fetuins antagonized the antiproliferative action of transforming growth factor (TGF)-β in a cell culture because fetuin binds directly to molecules of the TGF-β family, including bone morphogenic proteins. In addition, we recently reported that in rats, phosphorylated fetuin is a natural modulator of HGF (*13*), a broad-spectrum multifunctional cytokine (*41*), possibly through binding of its phosphorylated form to HGF. However, these biological functions of fetuin observed in *in vitro* experiments might not be applicable *in vivo* because relatively high concentrations of fetuin are required for these biological activities, *i.e.* 1-10 μg/ml of phosphorylated rat fetuin is needed for 50% inhibition of the effect of insulin on FaO cells (*11*), the dissociation constant (K_d) of fetuin, from TGF-β family proteins, is 10^{-6} to 10^{-7} M (*12*), and 1 μg/ml of phosphorylated rat fetuin inhibits HGF-stimulated DNA synthesis in rat hepatocytes by about 50% (*13*). The high concentrations of serum fetuin demonstrated in this study should overcome these problems.

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