Effect of protein restriction on the messenger RNA contents of bone-matrix proteins, insulin-like growth factors and insulin-like growth factor binding proteins in femur of ovariectomized rats

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It has been reported that loss of ovarian oestrogen after menopause or by ovariectomy causes osteoporosis. In order to elucidate the effect of dietary protein restriction on bone metabolism after ovariectomy, we fed ovariectomized young female rats on a casein-based diet (50 g/kg diet (protein restriction) or 200 g/kg diet (control)) for 3 weeks and measured mRNA contents of bone-matrix proteins such as osteocalcin, osteopontin and α1 type I collagen, insulin-like growth factors (IGF) and IGF-binding proteins (IGFBP) in femur. Ovariectomy decreased the weight of fat-free dry bone and increased urinary excretion of pyridinium cross-links significantly, although dietary protein restriction did not affect them. Neither ovariectomy nor protein restriction affected the content of mRNA of osteopontin and osteocalcin; however, ovariectomy increased and protein restriction extensively decreased the α1 type I collagen mRNA content in bone tissues. Ovariectomy increased IGF-I mRNA only in the rats fed on the control diet. Conversely, protein restriction increased and ovariectomy decreased the IGF-II mRNA content in femur. Furthermore, the contents of IGFBP-2, IGFBP-4 and IGFBP-5 mRNA increased, but the content of IGFBP-3 mRNA decreased in femur of the rats fed on the protein-restricted diet. In particular, ovariectomy decreased the IGFBP-2 mRNA content in the protein-restricted rats and the IGFBP-6 mRNA content in the rats fed on the control diet. These results clearly show that the mRNA for some of the proteins which have been shown to be involved in bone formation are regulated by both quantity of dietary proteins and ovarian hormones.

Bone: Insulin-like growth factor: Ovariectomy: Osteoporosis

Osteoporosis is a serious disease worldwide. Among many types of this disease, postmenopausal osteoporosis is observed frequently. Decreased plasma oestrogen concentration is believed to be its principal cause (Christiansen *et al.* 1982; Gallagher, 1990). Accordingly, ovariectomized animals have been used as a suitable model to study osteoporosis, because loss of ovarian oestrogen by ovariectomy causes a similar situation to menopause (Kalu, 1991). In general, rapid decreases in bone mass have been observed in ovariectomized rats (Wronski *et al.* 1985; Kalu *et al.* 1989). This decrease in bone mass is known as osteopenia which has been shown to be caused by raised bone turnover, due to increased bone resorption and the concomitant increase in bone formation (Wronski *et al.* 1986; Ismail *et al.* 1988); the raised bone turnover is depressed by oestrogen (Wronski *et al.* 1988*a*; Kalu *et al.* 1991). It is well known that bone resorption is performed by osteoclasts and bone formation by osteoblasts. Therefore, osteopenia is thought to be the result of the much increased activity of osteoclasts and less enhanced activity of osteoblasts by lack of ovarian oestrogen.

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Turner *et al.* (1990) reported that expression of osteocalcin, osteonectin, osteopontin and $\alpha1$ chain of type I collagen genes in periosteal osteoblasts of ovariectomized rats were down-regulated by treatment of the animals with diethylstilboestrol. This means that oestrogens regulate the gene expression of these proteins which have been presumed to play important roles in bone formation. Furthermore, some growth factors and cytokines were reported to be involved in regulation of bone metabolism (Manolagas & Jilka, 1992; Baylink *et al.* 1993; Canalis *et al.* 1993; Horowitz 1993). In particular, insulin-like growth factors (IGF) and their binding proteins (IGFBP), which could modulate IGF's actions, might play important roles in bone formation (Nilsson *et al.* 1994; Rosen *et al.* 1994). Finally dietary factors have been shown to be important in bone and Ca metabolism (LeRoith & Pimstone, 1973); however, the effect of dietary conditions on the changes in mRNA of bone-matrix proteins and some growth factors in bones has not been studied.

The present study was undertaken to investigate the effects of protein restriction on changes in the mRNA contents of osteopontin, osteocalcin, $\alpha1$ type I collagen, IGF-I, IGF-II and IGFBP in femur of ovariectomized rats.

MATERIALS AND METHODS
Animals

Female Wistar-strain rats (7 weeks old) were obtained from Charles River Japan (Kanagawa, Japan) and fed on a diet containing 200 g casein/kg diet (200C diet, Table 1) until the operation. The rats were given the diet from 09.00 to 17.00 hours and water *ad libitum*. The room was kept at 22° and the relative humidity at 60% with a 12-h light (08.00–20.00 hours) and 12 h dark (20.00–08.00 hours) cycle. After 4 d the rats were either ovariectomized bilaterally or sham-operated. Then the rats were divided into two dietary groups; one was those fed on the 200C diet (control diet) (initial body weight: sham-operated rats, 193 (SE 2) g, *n* 5; ovariectomized rats, 189 (SE 2) g, *n* 5) and the other was those fed on the 50 g casein/kg diet (50C diet, protein-restricted diet) (initial body weight: sham-operated rats, 186 (SE 5) g, *n* 5; ovariectomized rats, 190 (SE 5) g, *n* 5). The 50C diet was prepared by replacing the 150 g casein/kg and 2·5 g methionine/kg in the 200C diet by maize starch and addition of $CaHPO_4$, NaH_2PO_4 and Na_2HPO_4 to adjust the contents of Ca and P in the 200C diet (Table 1). The feed intake of the 200C diet group was paired to that of the 50C diet group of the same operation, i.e. the feed intakes of the ovariectomized and sham-operated rats fed on the 50C diet were measured and the same amounts of the 200C diet were fed to the paired rats on the next day. At 3 weeks later the rats were fed at 10.00 hours and killed at 11.30 hours by decapitation under pentobarbital anaesthesia (50 mg pentobarbital/kg body weight). The uterus was excised and weighed. Then, femurs were quickly excised and put into liquid N_2. These frozen femurs were stored at $-80°$ until the preparation of RNA. For the last 24 h before the rats were killed, urine samples were collected. The concentration of pyridinoline in urine was determined by an inhibition immunoassay method (Tanaka *et al.* 1993), based on that of Robins (1982).

Total RNA preparation from bone tissues

Total RNA was prepared from femurs according to the modified method of Chomczynski & Sacchi (1987), Nemeth *et al.* (1989) and Puissant & Houdebine (1990). After muscle and tendon were removed, cartilages at the ends of bones were further cut away from femurs in liquid N_2. The bones, except the cartilages, were dissected into fine pieces with scissors, also in liquid N_2. This frozen bone (we used one femur for one extraction) was put into 10 ml solution A containing 4 M-guanidinium thiocyanate, 25 mM-sodium citrate, pH 7·0, 5 g sarcosyl/l, 0·1 M-2-mercaptoethanol and 5 g antifoam A/l and quickly homogenized

with a Polytron homogenizer (Kinematica, Littau, Luzern, Switzerland). The homogenate was centrifuged at 7000 g for 2 h at 18°. The supernatant fraction was filtered through PTFE (pore size 0·5 μm, Corning, Ithaca, NY, USA). To the filtrate, 1 ml 2 M-sodium acetate, pH 4·0, 10 ml phenol which had been saturated with water, and 2 ml chloroform–isoamylalcohol (49:1, v/v) were added and the mixture was shaken vigorously at each addition. The mixture was centrifuged at 10000 g for 10 min at −20°. The water phase was saved and mixed with an equal volume of isopropanol. The mixture was kept at 4° for more than 2 h in order to precipitate RNA. The precipitated RNA was centrifuged at 3000 g for 10 min at 4°. The precipitate was suspended in 2 ml 4 M-LiCl. The suspension was pipetted repeatedly to extract polysaccharides, then centrifuged at 3000 g for 10 min at 4°. The precipitate was redissolved in 500 μl of a solution containing 10 mM-Tris-HCl, pH 7·5, 1 mM-EDTA, and 5 g SDS/l. This solution was again mixed with 500 μl chloroform–isoamylalcohol (49:1, v/v) and centrifuged at 3000 g for 10 min at 4°. The water phase was mixed with 500 μl isopropanol and 50 μl 2 M-sodium acetate, pH 5·0. The precipitated RNA in the mixture was recovered by centrifugation at 15000 g for 10 min at 4°, dried *in vacuo*, and dissolved in distilled water pretreated with diethylpyrocarbonate by heating at 37° for 12 h. By determining the absorbance at 260 and 280 nm, the purity of RNA was confirmed and the concentration of RNA in the solution was determined.

To prepare poly(A)$^+$ RNA for the template of reverse transcriptase–polymerase chain reaction (RT–PCR), we purified it from total RNA using Oligotex®-dT30 (Japan Roche, Tokyo, Japan) according to the manufacturer's instructions.

Probes for Northern hybridization

The probes for Northern hybridization were complementary DNA (cDNA) of rat osteopontin, rat osteocalcin, rat α1 type I collagen, rat IGF-I, rat IGF-II, and rat IGFBP-1 to IGFBP-6 (Table 2).

Rat osteopontin cDNA which covers the nucleotides 366–933 of the cDNA clone reported previously (Oldberg *et al.* 1986) was amplified by a RT–PCR technique. The nucleotide sequence of this amplified cDNA corresponds to His[97]-His[285] of osteopontin protein. Rat osteocalcin cDNA, which covers the nucleotides 61–366 of the cDNA clone reported previously (Celeste *et al.* 1986), was also amplified by the RT–PCR technique. This cDNA encodes Ser[−5]-Val[50], which corresponds to a part of the signal peptide and whole mature protein, and a part of the 3′-non-coding region of the osteocalcin gene. At first, primer oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems Model 391 PCR-MATE, Foster City, CA, USA). For rat osteopontin cDNA the sense primer was 24mer (5′-GGTACCATGCAGAGAGCGAGGATT-3′) and the antisense primer was 24mer (5′-GAATTCATGGCTGTGAAACTCGTG-3′). For rat osteocalcin cDNA the sense primer was 20mer (5′-TCTCTGCTCACTCTGCTGGC-3′) and the antisense primer was 21mer (5′-TCCAGGGCAACACATGCCCTA-3′). These nucleotides were purified by PAGE.

Using them, RT–PCR was performed as follows. In the case of rat osteopontin cDNA we prepared and cultured osteoblast cells from rat calvaria according to the method of Wong & Cohn (1974), and total RNA was prepared from osteoblast cells in primary cultures as described previously (Miura *et al.* 1992). This total RNA (5 μg) as a template was mixed with 50 pmol 3′-primer in 50 mM-Tris-HCl pH 8·3, 100 mM-KCl and 10 mM-MgCl$_2$, and heated at 78° for 5 min. The mixture was cooled to 4° for 5 min and was mixed with 10 mM-dithiothreitol, 2U RNase inhibitor (Takara, Kyoto, Japan), 1 mM each deoxynucleotide and 2 μl of reverse transcriptase RAV-2 (Takara, Kyoto, Japan) at 42° for 1 h. The PCR reactions were performed with AmpliTaq DNA polymerase (*EC* 2.7.7.7; Takara, Kyoto, Japan) according to the manufacturer's instructions. First, the mixture was

Table 1. *The composition of the experimental diets (g/kg)*

	Diet...	200C	50C
	Protein level (g/kg diet)...	200	50
	Casein* (g)	235·5	58·8
	β-Maize starch (g)	559·2	727·9
	Soyabean oil (g)	50	50
	Mineral mixture (g)†	40	40
	Vitamin mixture (g)‡	10	10
	Cellulose (g)	100	100
	Choline chloride (g)	2	2
	L-Methionine (g)	3·3	0·8
	$CaHPO_4.2H_2O$ (g)§	—	0·16
	$NaH_2PO_4.2H_2O$ (g)§	—	3·1
	$Na_2HPO_4.12H_2O$ (g)§	—	7·2

* Crude protein 850 g/kg.

† The mineral mixture was prepared according to the American Institute of Nutrition (1977).

‡ The vitamin mixture was purchased from Oriental Yeast Co., Tokyo, Japan. Its composition was (mg/kg): retinyl acetate 172, cholecalciferol 2·5, α-tocopheryl acetate 5000, menadione 5200, thiamin hydrochloride 1200, riboflavin 4000, pyridoxine hydrochloride 800, cyanocobalamin 0·5, L-ascorbic acid 30000, biotin 20, pteroylmonoglutamic acid 200, calcium pantothenate 5000, p-amino benzoic acid 5000, nicotinic acid 6000, inositol 6000, choline chloride 200000.

§ To adjust the contents of Ca and P in the 50C diet to those in the 200C diet, $CaHPO_4$, NaH_2PO_4 and Na_2HPO_4 were added to the 50C diet.

Table 2. *Complementary DNA (cDNA) probes used for Northern hybridization analyses*

cDNA probe	Nucleotide numbers included in the probe	Amino acid sequence which the probe recognizes	Reference
Rat osteopontin	bp 366–933	His[97]-His[284]	Oldberg *et al.* 1986
Rat osteocalcin	bp 61–366	Ser[-5]-Val[50]	Celeste *et al.* 1986
Rat α1 type I collagen	*Pst*I/*Bam*HI fragment	Part of the triple helical region	Genovese *et al.* 1984
Rat IGF-I	bp 1–738	Met[-48]-Met[105]	Kato *et al.* 1990
Rat IGF-II	*Pst*I fragment	Arg[38]-Gln[156]	Whitfield *et al.* 1984
Rat IGFBP-1	bp 123–848	Pro[11]-Ile[252]	Takenaka *et al.* 1991
Rat IGFBP-2	bp 614–957	Arg[118]-His[231]	Brown *et al.* 1989
Rat IGFBP-3	bp 451–940	Glu[139]-Gln[291]	Takenaka *et al.* 1991
Rat IGFBP-4	bp 502–703	Glu[86]-Glu[152]	Shimasaki *et al.* 1990
Rat IGFBP-5	bp 558–1201	Ile[-17]-Asn[198]	Shimasaki *et al.* 1991*b*
Rat IGFBP-6	bp 308–508	Pro[77]-Gly[142]	Shimasaki *et al.* 1991*a*

incubated at 94° for 5 min. Then thirty cycles consisted of the denaturation step at 94° for 1 min, the annealing step at 50° for 1 min and the extension step at 72° for 2 min. The last cycle consisted of 94° for 1 min, 50° for 1 min, and 72° for 10 min. From this reaction mixture we got 576 bp cDNA which was cloned to M13 phage vector followed by sequencing. In the case of rat osteocalcin cDNA the poly A^+ RNA (5 μg) prepared from rat femur as a template was used for RT–PCR as described previously. We amplified 306 bp cDNA and identified it as rat osteocalcin cDNA by sequencing.

The cDNA of rat IGF-1, rat IGFBP-1 and rat IGFBP-3 were obtained as described previously (Kato *et al.* 1990; Takenaka *et al.* 1991). Rat α1 type I collagen cDNA

(Genovese *et al.* 1984) was kindly given by Dr H. Shikata of Meikai University (Saitama, Japan) and cDNA of rat IGF-II (Whitfield *et al.* 1984), rat IGFBP-2, rat IGFBP-4, rat IGFBP-5 and rat IGFBP-6 (Lemozy *et al.* 1994) were generously donated by Dr A. J. D'Ercole of the University of North Carolina at Chapel Hill (NC, USA).

All these probes were labelled with [^{32}P]deoxycytidine triphosphate (dCTP) (111 TBq/ mmol; Amersham, Bucks.) using a Takara Random Primer DNA Labelling Kit (Takara, Kyoto, Japan) according to the manufacturer's instructions.

Northern hybridization analysis

Northern hybridization analysis was performed as described previously (Miura *et al.* 1992). Total RNA (5 μg for α1 type I collagen mRNA, 20 μg for osteocalcin and osteopontin mRNA or 40 μg for IGF-I, IGF-II and each IGFBP mRNA as optimal amounts) was dissolved in 20 μl of a solution containing 500 ml deionized formamide/l, 30 ml formaldehyde/l, 80 μg ethidium bromide/ml in 3-(*N*-morpholino)propanesulphonic acid (MOPS) buffer solution (Rosen *et al.* 1990) and heated at 55° for 15 min. The denatured RNA was quickly electrophoresed in a gel containing 15 g agarose/l and 2·2 M-formaldehyde. After electrophoresis the amount of RNA in the gel was confirmed to be equivalent by a transilluminator. The RNA in the gel was transferred to a nitrocellulose membrane and each mRNA was detected on the blot using labelled cDNA as described previously (Miura *et al.* 1992; Takenaka *et al.* 1993). Each band was quantified by a bio-imaging analyser system, Fujix Bas 2000 or 3000 (Fuji Film Co., Tokyo, Japan).

Statistical analysis

The results were analysed statistically by two-way ANOVA (Snedecor & Cochran, 1967).

RESULTS

The effects of ovariectomy and dietary protein restriction on final body weight, feed efficiency, uterus weight, fat-free dry bone weight and urinary excretion of pyridinium cross-links

Table 3 shows the body-weight changes in the groups of rats. Ovariectomized rats gained more weight than the sham-operated rats, partly because the ovariectomized rats ate more of both the 50C and 200C diets. Since each of the sham-operated or ovariectomized rats fed on the 50C diet was paired to one of the corresponding group fed on the 200C diet and the rats of the paired groups ate all the offered feed, variations in feed intake were also reproduced in the paired group. Furthermore, feed efficiency was better in ovariectomized rats than sham-operated ones. As shown in Table 3, the effect of ovariectomy was also confirmed by examination of the degenerated uterus at the time of death. Ovariectomized rats showed lower fat-free dry bone (FFDB) weight; however, there was no significant difference between the dietary groups in FFDB weight. Furthermore, ovariectomy caused an increase in pyridinoline excretion in the urine (Table 3).

The effect of ovariectomy and dietary protein restriction on mRNA of bone-matrix proteins in femur

Fig. 1 and Table 4 show the effect of ovariectomy and dietary protein restriction on the amount of mRNA of osteopontin, osteocalcin and α1 type I collagen in femur. Neither ovariectomy nor protein restriction affected the content of mRNA of osteopontin and osteocalcin in femur. Therefore, we conclude that ovariectomy and dietary protein restriction do not affect the osteocalcin mRNA content in femur, at least under the present experimental conditions. By comparison, ovariectomy increased, and protein

Table 3. *Final body weight (BW), feed intake, feed efficiency, uterus weight, fat-free dry bone weight and urinary excretion of pyridinium cross-links in ovariectomized rats fed on diets containing 200 (200C) or 50 g casein/kg (50C)*†

(Mean values for five rats per group with their pooled standard error)

Diet...	200C		50C			Statistical significance of effect of:	
Treatment...	Sham operated	Ovariectomized	Sham operated	Ovariectomized	SEM	Diet	Ovariectomy
BW gain (g)	49	75	40	60	5	*	**
Feed intake (g/d)‡	14·9	17·1	14·9	17·1	0·6	NS	**
Feed efficiency§	17·2	23·1	14·0	18·5	1·1	**	**
Uterus weight (g)	0·38	0·09	0·41	0·11	0·03	NS	**
Fat-free dry bone weight (mg/g BW)	1·54	1·41	1·56	1·43	0·02	NS	**
Urinary excretion of pyridinium cross-links (mg/d)	2·2	4·3	2·6	4·3	0·6	NS	**

* $P < 0.05$, ** $P < 0.01$.
† For details of diets and procedures, see Table 1 and p. 812.
‡ Daily feed intakes of the 200C and 50C groups were paired.
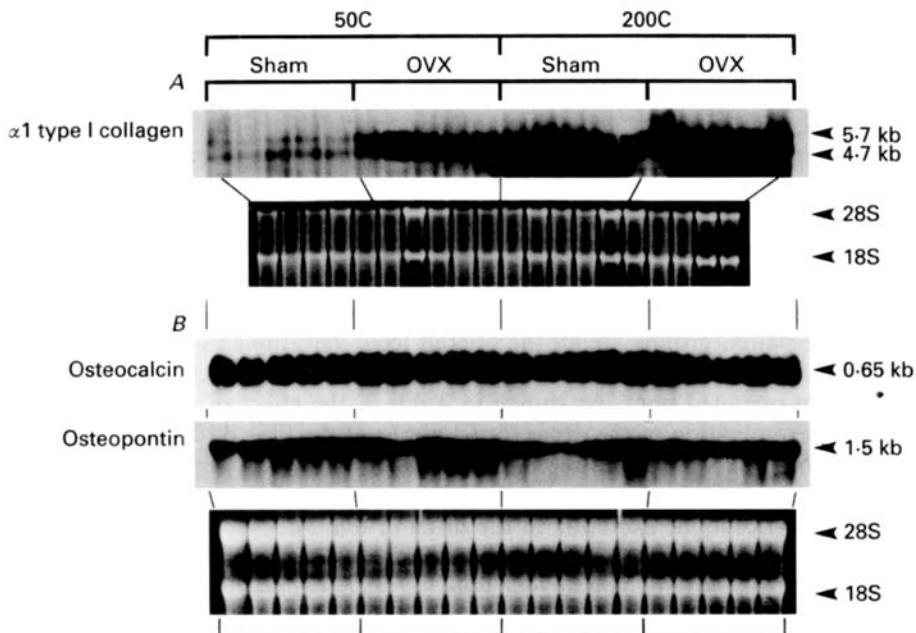§ (g BW gain/g feed intake) × 100.

Fig. 1. Northern blot analysis of α1 type I collagen, osteopontin, and osteocalcin messenger RNA (mRNA) in femur from sham-operated (Sham) or ovariectomized (OVX) rats fed diets containing 200 (200C) or 50 g casein/kg (50C). RNA was extracted from femur of Sham or OVX rats fed on the 50C or 200C diets for 3 weeks. In the case of α1 type I collagen, a 5 μg portion of total RNA from each rat was applied to each lane (A) and in the case of osteopontin and osteocalcin, a 20 μg portion of total RNA was applied (B). The size of each mRNA is indicated on the right. Bands of ribosomal RNA stained by ethidium bromide (bottom of each figure, sizes shown on the right) demonstrate equal amounts of total RNA in each lane.

Table 4. *Effect of ovariectomy on the messenger RNA contents of bone matrix proteins in femur of rats fed on diets containing 200 (200C) or 50 g casein/kg (50C)†*

(The results of Northern blot hybridization analyses shown in Fig. 1 were determined quantitatively using a Fujix Bas 2000 or 3000 system (Fuji Film Co.). Values are means for five rats expressed as the relative intensity of the bands taking the mean of sham operated 200C group as 100)

Diet...	200C		50C			Statistical significance of effect of:	
Treatment...	Sham operated	Ovariectomized	Sham operated	Ovariectomized	SEM	Diet	Ovariectomy
Osteopontin	100·0	143·5	149·3	132·7	14·6	NS	NS
Osteocalcin	100·0	96·4	111·7	117·3	13·1	NS	NS
α1 Type 1 collagen							
5·7 kb	41·0	67·6	19·9	41·1	5·1	**	**
4·7 kb	59·0	81·8	23·8	44·9	5·3	**	**
Total	100·0	149·4	43·7	86·0	9·5	**	**

** $P < 0.01$.

† For details of diets and procedures, see Table 1 and pp. 812–815.
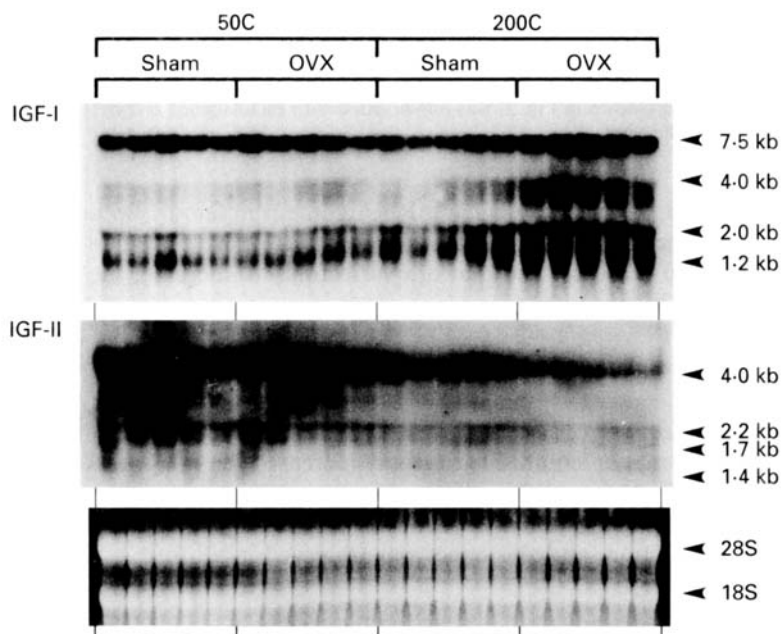
Fig. 2. Northern blot analysis of insulin-like growth factor-I (IGF-I) and IGF-II mRNA in femur from sham-operated (Sham) or ovariectomized (OVX) rats fed on diets containing 200 (200C) or 50 g casein/kg (50C). RNA was extracted from femur of Sham or OVX rats fed on the 50C or 200C diets for 3 weeks. A 40 μg portion of total RNA from each rat was applied to each lane. The size of each mRNA is indicated on the right. Bands of ribosomal RNA stained by ethidium bromide (bottom, sizes shown on the right) demonstrate an equal amount of total RNA in each lane.
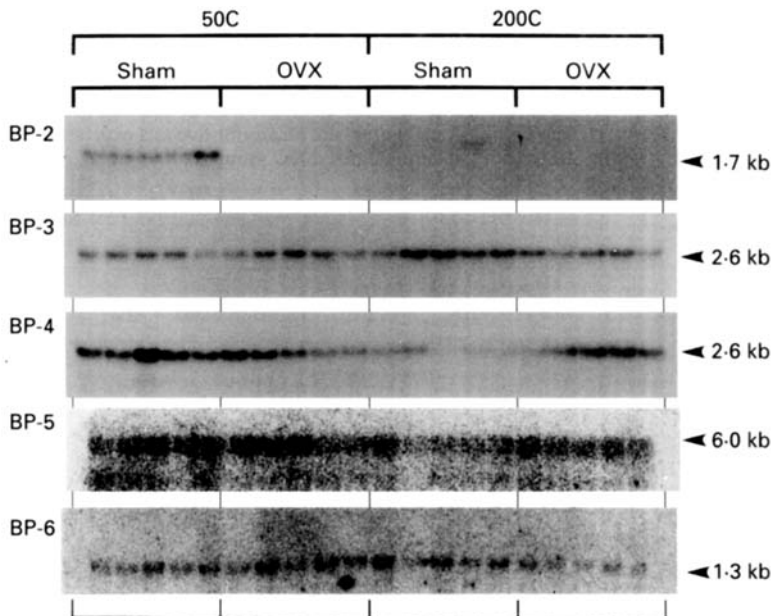
Fig. 3. Northern blot analysis of insulin-like growth factor-binding protein-2 (IGFBP-2), IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6 messenger RNA (mRNA) in femur from sham-operated (Sham) or ovariectomized (OVX) rats fed on diets containing 200 (200C) or 50 g casein/kg (50C). The membrane prepared for analysis of IGF-I and IGF-II mRNA (shown in Fig. 2) was rehybridized with each labelled IGFBP probe. The size of each mRNA is indicated on the right.

restriction decreased, α1 type I collagen mRNA content in femur. Because the interaction of these two treatments was not statistically significant, we conclude that the effect of protein restriction to decrease, and that of ovariectomy to increase, the α1 type I collagen mRNA content are independent.

The effect of ovariectomy and dietary protein restriction on mRNA of insulin-like growth factors in femur

Fig. 2 shows the effects of ovariectomy and dietary protein restriction on the IGF-I and IGF-II mRNA contents in femur. Ovariectomy increased the IGF-I mRNA content in the rats fed on the 200C diet, and this effect was most noticeable in the 4·0, 2·0 and 1·2 kb species. Conversely IGF-II mRNA content was decreased by ovariectomy and increased by dietary protein restriction, and this effect was observed in all four of the IGF-II mRNA species.

The effects of ovariectomy and dietary protein restriction on mRNA of insulin-like growth factor-binding proteins in femur

IGFBP-1 mRNA was not detected in femur even in the protein-restricted and ovariectomized rats (results not shown). Fig. 3 and Table 5 show the effects of ovariectomy and protein restriction on femur IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 mRNA contents. Protein restriction increased the IGFBP-2 mRNA content and ovariectomy reduced it considerably in both diet groups. As for the IGFBP-3 mRNA, its content was significantly reduced by protein restriction. Conversely, IGFBP-4 and IGFBP-5 mRNA contents in femur increased during protein restriction. IGFBP-6 mRNA in the

Table 5. *Effect of ovariectomy on the messenger RNA contents of insulin-like growth factor-binding proteins (IGFBP) in femur of rats fed on diets containing 200 (200C) or 50 g casein/kg (50C)†*

(The results of Northern blot hybridization analyses shown in Fig. 3 were determined quantitatively using a Fujix Bas 2000 or 3000 system (Fuji Film Co.). Values are means for five rats expressed as the relative intensity of the bands taking the mean of sham operated 200C group as 100)

Diet...	200C		50C			Statistical significance of effect of:		
Treatment...	Sham operated	Ovariectomized	Sham operated	Ovariectomized	SEM	Diet (D)	Ovariectomy (O)	O × D
IGFBP-2	100·0	54·7	273·3	19·0	16·7	**	**	**
IGFBP-3	100·0	87·2	69·8	70·7	6·2	**	NS	NS
IGFBP-4	100·0	223·8	270·1	185·4	26·1	*	NS	**
IGFBP-5	100·0	125·4	157·9	146·3	10·8	**	NS	NS
IGFBP-6	100·0	64·7	101·1	119·7	12·6	*	NS	*

* $P < 0.05$, ** $P < 0.01$.
† For details of diets and procedures, see Table 1 and pp. 812–815.

femur was also increased by protein restriction and furthermore ovariectomy significantly decreased its content in the rats fed on the control diet.

DISCUSSION

As shown in Table 3, the effect of ovariectomy was confirmed by examination of the degenerated uterus and lower FFDB weight at the time of death; however, there was no significant difference between the dietary groups in FFDB weight. The fact that ovariectomy also increased excretion of pyridinium cross-links in the urine (Table 3) suggested that the decrease in bone mass in ovariectomized rats was caused by accelerated turnover of bone metabolism through lack of ovarian oestrogens. In these rats, ovariectomy and protein restriction clearly affected the mRNA content of the bone-matrix protein for α1 type I collagen and IGF, which have been presumed to play important roles in bone formation.

In the present study we developed a modified method to extract the total RNA from the homogenate of femurs by slight modification of the method of Chomczynski & Sacchi (1987), Nemeth *et al.* (1989) and Puissant & Houdebine (1990). This modification involved extraction of the total RNA from whole femurs except the cartilages at the end of the bones, described on pp. 812–813. Since we removed the epiphyseal growth plate where active bone formation is observed in growing rats, we believe that our results reflect changes in the whole bone, showing primarily the activity of trabecular bone turnover. Turner *et al.* (1990) reported that a treatment with diethylstilboestrol decreased the contents of mRNA of α1 type I collagen, osteopontin and osteocalcin in the periosteal cells obtained from femur of ovariectomized rats by collagenase (*EC* 3.4.24.3) digestion. Westerlind *et al.* (1993) reported similar results in the cancellous bone of ovariectomized rats and recently showed that ovariectomy increased α1 type I collagen and osteocalcin mRNA in bone (Westerlind *et al.* 1994). Our present results are consistent with their observations in the case of α1 type I collagen and further show the effect of dietary protein restriction on the changes in mRNA of these bone-matrix proteins. In particular, the observations that both ovariectomy and protein restriction affected the mRNA contents of the proteins produced by osteoblasts could be very important.

IGF-I mRNA was increased by ovariectomy only in the high-protein group. Ovariectomy also increased $\alpha 1$ type I collagen mRNA, despite the fact that it also reduced FFDB weight, whilst dietary protein restriction decreased the $\alpha 1$ type I collagen mRNA. These apparently conflicting changes could possibly be explained by the following hypothesis. Following ovariectomy, osteoclast activity and bone resorption are enhanced. At the same time, osteoblast activity and bone formation are enhanced (Wronski *et al.* 1988*a*). Gain or loss of bone tissue is the result of the balance between these two processes. This activation of osteoblasts could be the compensatory effect of the enhanced bone resorption. Collagen $\alpha 1$ type I has been shown to be synthesized in bone primarily by osteoblasts (Sandberg & Vuorio, 1987; Sandberg *et al.* 1988, 1989). Therefore, the increase in $\alpha 1$ type I collagen mRNA is well explained by the increased activity of osteoblasts.

The activity of osteoblasts, including the possibly enhanced differentiation of progenitor cells to osteoblasts, has been shown to be regulated by many factors (Baylink *et al.* 1993; Canalis *et al.* 1993). IGF-I is thought to be one of these factors which can regulate the activity of osteoblasts to stimulate their proliferation and the synthesis of bone proteins (Canalis, 1980; McCarthy *et al.* 1989; Thiebaud *et al.* 1990). Also, many researchers have reported that IGF are produced locally in bone tissues (Stracke *et al.* 1984; Canalis *et al.* 1988) and that their production is regulated by oestrogens (Ernst *et al.* 1989; Gray *et al.* 1989). These observations are possibly inconsistent with our results that ovariectomy increased the content of IGF-I mRNA in femur. Therefore, the increase in IGF-I mRNA by ovariectomy may mean the involvement of this hormone in the compensatory activity of bone resorption by stimulation of osteoblasts. If this is the case, IGF-I produced in bone probably works on the bone tissue itself in an autocrine or paracrine fashion. The fact that the increase in IGF-I mRNA is only observed in well-fed rats may mean that the compensatory activation of osteoblasts is impaired in protein-restricted rats. We and others have shown that quantity and quality of dietary proteins affect the plasma IGF-I concentration and content of IGF-I mRNA in rat liver (Prewitt *et al.* 1982; Straus & Takemoto, 1990; Takahashi *et al.* 1990; Miura *et al.* 1992). So the content of IGF-I mRNA in femur could be controlled by a similar mechanism to that in liver in response to dietary proteins.

Unlike the case of IGF-I, the femur mRNA content of IGF-II decreased in the ovariectomized rats, but clearly increased in the protein-restricted rats; however, the physiological significance of the increase in IGF-II mRNA in bone under protein restriction and the decrease in ovariectomy remain to be elucidated.

Messenger RNA of IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 were found in femur in the present study. Our results confirm the previous observations that rat osteoblasts express these five IGFBP (Schmid *et al.* 1992; Birnbaum & Wiren, 1994). The content of each IGFBP mRNA in femur was regulated by protein restriction and ovariectomy in different ways, as we showed here. We and others have reported that plasma concentration and mRNA content in liver of each IGFBP are also differently regulated (Donovan *et al.* 1991; Thissen *et al.* 1991; Umezawa *et al.* 1991; Takenaka *et al.* 1993). However, the effects of protein restriction and ovariectomy on the contents of these IGFBP in femur were different from those on the mRNA contents of these IGFBP in liver which we will report in a following paper (Y. Higashi, A. Takenaka, S.-I. Takahashi and T. Noguchi, unpublished results). Notably, an increase in IGFBP-2 mRNA under protein restriction was not observed in the femur of ovariectomized rats. This may mean that IGFBP-2 gene expression is mainly regulated by oestrogens in femur. In the case of IGFBP-4, mRNA content responded to protein restriction in femur, but not in liver. At present, we do not have any information concerning the oestrogen response elements in the genes of any IGFBP except IGFBP-6 (Zhu *et al.* 1993). The reason for the different response

between the tissues is not explained yet; however, the distinctive regulation of IGFBP in femur could be important in controlling bone formation. As described previously, IGFBP have been shown to inhibit or enhance IGF's action in cell-specific ways. In osteoblast cultures it has been reported that IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 inhibit IGF's action and IGFBP-5 stimulates the activity of IGF (Mohan *et al.* 1989; Campbell & Novak, 1991; Feyen *et al.* 1991; Andreas & Birnbaum, 1992). Furthermore, the role of IGFBP-6 is not clear. Taken together, regulated production of IGFBP in response to oestrogens or protein restriction in femur could affect the availability of IGF and as a result, bone formation could be controlled through modulated IGF activities.

In summary, the present results show that both ovariectomy and protein restriction can affect the mRNA contents, in femur, of some proteins which have been presumed to be important physiologically in bone formation. The results suggest that protein restriction after menopause should be carefully avoided, because it may depress the activity of bone formation by osteoblasts.

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