

The effect of bovine whey protein on ectopic bone formation in young growing rats

Owen Kelly¹, Siobhan Cusack¹ and Kevin D. Cashman^{1,2*}

¹Department of Food and Nutritional Sciences and

²Department of Medicine, University College, Cork, Republic of Ireland

(Received 7 March 2002 – Revised 20 March 2003 – Accepted 14 May 2003)

The beneficial effect of bovine whey protein (WP) on bone metabolism has been shown in adult human subjects and ovariectomised rats. However, its effect on bone formation in earlier life, particularly during periods of bone mineral accrual, has not been investigated. Twenty-one male rats (4 weeks old, Wistar strain) were randomised by weight into three groups of seven rats each and fed *ad libitum* on a semi-purified low-Ca diet (3.0 g Ca/kg diet) containing 0 (control), 10 (diet WP1) or 20 (diet WP2) g bovine WP/kg for 47 d. On day 34 of the dietary intervention, all rats had two gelatine capsules containing demineralised bone powder implanted subcutaneously in the thorax region (a well-established *in vivo* model of ectopic bone formation). At 14 d after implantation, alkaline phosphatase activity (reflective of bone formation) in the bone implants from animals fed WP1 and -2 diets was almost 2-fold ($P < 0.01$) that of control animals. Insulin-like growth factor (IGF)-I mRNA levels were about 3-fold ($P < 0.05$) higher in implants from animals fed the WP diets compared with those from control animals. Serum- and urine-based biomarkers of bone metabolism and bone mineral composition in intact femora were unaffected by WP supplementation. In conclusion, the present findings suggest that bovine WP can enhance the rate of ectopic bone formation in young growing rats fed a Ca-restricted diet. This effect may be mediated by an increased synthesis of IGF-I in growing bone. The effect of WP on bone formation warrants further investigation.

Bone formation: Ectopic bone: Whey protein: Rats

Whey protein (WP) is a major component of the protein fraction in bovine milk. Milk contains 0.55 g WP/l and the major protein fractions are α -lactalbumin (58%), β -lactoglobulin (20%), immunoglobulin (13%) and serum albumin (7%) (Ito, 1991). Takada *et al.* (1997a,c) reported that increasing the dietary intake of bovine milk WP (either as the intact or fractionated protein) was effective in increasing the breaking strength and breaking energy of bone, as well as increasing the total amino acid content (especially that of collagen-specific amino acids) in the bone of ovariectomised rats. However, the mechanism of these effects of WP on bone is unclear. For example, while bone strength of the ovariectomised rats was increased by supplementation of the diet with WP, Ca absorption or retention, and bone mineral content were unaffected (Takada *et al.* 1997a,c).

WP has been shown to stimulate the proliferation and differentiation of murine osteoblastic MC3T3-E1 cells *in vitro* and to increase the total protein and hydroxyproline contents in these cells (Takada *et al.* 1996). Furthermore, Takada *et al.* (1997b) examined the effect of increasing the concentration of dietary WP on bone resorption using the pre-existing and newly formed osteoclast model and the haemopoietic blast cell culturing system. They

found that WP suppressed osteoclast-mediated bone resorption and osteoclastic cell formation (Takada *et al.* 1997b). Recently, the basic fraction of WP (milk basic protein) has been shown to suppress the elevated rate of bone resorption and bone loss associated with ovariectomy in adult rats (Toba *et al.* 2000). Moreover, in two recent dietary intervention studies in human subjects, milk basic protein has been shown to increase calcaneal bone mineral density and reduce bone resorption in healthy adult women (Aoe *et al.* 2001), and to increase bone formation while suppressing bone resorption in healthy adult men (Toba *et al.* 2001). However, the effect of WP on skeletal metabolism in earlier life has not been investigated.

Several highly bone-active molecules have been isolated from WP, in particular, insulin-like growth factor (IGF)-I (Ballard *et al.* 1982), the most abundant growth factor in bone. IGF-I has potent anabolic effects on growing skeletal tissue, e.g. it enhances chondrocyte proliferation in the growth plate; in bone tissue it stimulates osteoblast proliferation and differentiation and matrix formation, including synthesis of type I collagen and other components (Price *et al.* 1994). Takada *et al.* (1996) found that the stimulatory effect of WP on osteoblastic cell proliferation could be neutralised by IGF-I and -II antibodies,

Abbreviations: IGF, insulin-like growth factor; WP, whey protein.

* **Corresponding author:** Professor Kevin D. Cashman, fax +353 21 4270244, email k.cashman@ucc.ie

suggesting that these polypeptide growth factors were involved. Schurch *et al.* (1998) reported that milk protein supplementation increases serum IGF-I concentrations, as well as reducing the rate of post-fracture bone loss in elderly patients. However, whether changes in circulating concentrations of the IGF-I reflect local (bone tissue) concentrations is uncertain, and the amount in bone tissue may be more important for bone formation (Rodan & Rodan 1995).

The objectives of the present study were first to investigate the effect of dietary (bovine) WP on bone formation using an *in vivo* ectopic bone formation model in young growing rats (i.e. subcutaneous implants of demineralised bone particles), and second, in parallel, to determine the influence of dietary WP on anatomic whole-body bone turnover and on bone composition in the same young growing animals. In addition, the influence of dietary WP supplementation on IGF-I expression in ectopic bone was investigated. The advantages and disadvantages of the ectopic bone formation model have been discussed previously (Sinha *et al.* 1988a). A major advantage, however, is that in this model effects on bone formation due to changes in diet are apparent much more rapidly than in total bone (Sinha *et al.* 1988b). In addition, it has been suggested that subtle changes in bone formation, as assessed by biochemical markers and that may not be measurable in total bone, are detectable in bone implant models (Sinha *et al.* 1988a,b).

Materials and methods

Preparation of rat diets

A slightly modified AIN-93G purified diet (recommended for growing rats by the American Institute of Nutrition; Reeves *et al.* 1993) was used in the present study (Table 1). The Ca content of the diet was reduced from the recommended 5.0 to 3.0 g Ca/kg diet to create a low-Ca diet.

Experimental design

Twenty-one male rats, 4 weeks old, Wistar strain (average weight 77.2 g), obtained from the Biological Services Unit, University College, Cork, Republic of Ireland, were randomised by weight into three groups of seven rats each. One group was fed *ad libitum* on the basal diet (AIN-93G) as outlined in Table 1, while the second and third group were fed diets similar to the basal diet in every respect except that 10 and 20 g casein component/kg was replaced by dietary WP (Bipro[®]; Mitchelstown Isolates Ltd, Mitchelstown, Cork, Republic of Ireland) (WP1 and -2 respectively). Rats were housed individually in metabolism cages with a grid floor and a facility for separate collection of faeces and urine. Feed was provided *ad libitum* at 17.00 hours each day and all animals were given distilled water *ad libitum* for the duration of the study. Rats were weighed weekly and examined each day for general condition.

After 34 d on the respective diets, all animals were anaesthetised with diethyl ether and their abdominal area shaved of hair. A 10 mm incision was made in the

Table 1. Composition of the diet*

Ingredient	Content (g/kg)
Casein	200.000†
L-Cystine	3.000
Maize starch	523.100
Sucrose	99.986
Fibre	50.000
Soyabean oil	70.000
AIN-93G modified mineral mix‡	35.000
AIN-93G vitamin mix§	10.000
Calcium carbonate	7.500
Choline chloride	1.400
<i>tert</i> -Butylhydroquinone	0.014

* Modified AIN-93G diet (Reeves *et al.* 1993).

† Representing diets containing (per kg): 200 and 0 g casein and whey protein respectively (control diet); 190 and 10 g casein and whey protein respectively (WP1 diet); 180 and 20 g casein and whey protein respectively (WP2 diet).

‡ Contained (mg/kg mix): K 3600, Cl 1571, P 1561, Na 1019, Mg 507, S 300, Fe 35, Zn 30, Mn 10, Cu 6, Si 5, Cr 1, F 1, B 0.5, Ni 0.5, I 0.2, Mo 0.15, Se 0.15, Li 0.1, V 0.1.

§ Contained (per kg mix): DL- α tocopheryl acetate 15 g, nicotinic acid 3 g, cyanocobalamin 2.5 g, calcium pantothenate 1.6 g, retinyl palmitate 1.2 g, cholecalciferol 1.0 g, pyridoxine hydrochloride 700 mg, thiamin hydrochloride 600 mg, riboflavin 600 mg, folic acid 200 mg, phyloquinone 75 mg, D-biotin 20 mg, sucrose 973.58 g.

epidermis at each side of the abdomen, just below the rib cage, under sterile conditions, and a pocket was prepared in each side by blunt dissection technique. A small gelatine capsule, containing demineralised bone powder (see later), was inserted subcutaneously into each of these surgically prepared pockets. Incisions were closed with metallic skin clips and the day of implantation designated as day 0. The implants remained in place for 14 d.

Urine samples (24 h) were collected for each animal over the last 3 d of the study in vessels covered with Al foil to prevent degradation by light of the pyridinium crosslinks. The urine samples for each animal were pooled and the volumes recorded. Portions of the pooled urine samples were acidified with 12 M-HCl (225 μ l/100 ml urine) and stored at -20°C until required for analysis.

Fourteen days after insertion of subcutaneous bone implants (and 48 d in total on the respective diets), all animals were anaesthetised with diethyl ether and exsanguinated by cardiac puncture. Blood drawn from the heart was processed to obtain serum, and this was immediately stored at -70°C until required. Final body weights were recorded and femora and implants were harvested. Femora were cleaned of adhering soft tissue. The distal epiphyses of the left femurs were removed and immediately placed in Al foil and immersed in liquid N₂. These were subsequently stored at -80°C until required for analyses for bone alkaline and acid phosphatases. The right femora were stored at -20°C until required for determination of femur length, mass, volume, density and mineral content. Bone implants were cleaned of adhering soft tissue, immersed in liquid N₂ and were subsequently stored at -80°C until required for alkaline and acid phosphatase and mRNA analysis.

Experimental techniques

Preparation of bone implants. Ten adult male rats, Wistar strain, obtained from the same breeder, were

anaesthetised with diethyl ether and killed. The femora and tibiae were excised, the extremities were removed with a saw and the bone marrow was flushed with distilled water. The diaphyses were scrubbed with a stiff brush and the adherent soft tissues were removed meticulously. The bones were washed with copious amounts of distilled water for 2.0 h, absolute ethanol for 1.0 h and diethyl ether for 0.5 h as described by Sinha *et al.* (1988*a,b*). The washed bones were dried overnight at room temperature (about 21°C). The cleaned dehydrated diaphyseal shafts were immersed in liquid N₂ and pulverised with hammer blows. The powder was sieved to contain particles of 70–425 µm diameter. The powder was demineralised in 0.5 M-HCl for 3 h. The mixture was centrifuged at 4500 g for 15 min and the precipitate washed in distilled water for 2.0 h, in absolute ethanol for 1.0 h, in diethyl ether for 0.5 h and the demineralised bone powder dried at room temperature. Demineralisation was confirmed by measurement of the Ca and inorganic P content of the powder (see later). Sterile gelatine (no. 5) capsules were filled with 30 mg demineralised bone powder and stored at room temperature in a sterile plastic tube until required for implantation.

Urinary pyridinoline and deoxypyridinoline. Pooled urine samples for each animal were analysed in duplicate using a three-step procedure. Urine was first hydrolysed with an equal volume of 12 M-HCl at 110°C for 18 h, the crosslinks were then extracted by CF1 cellulose chromatography with the use of an internal standard (acetylated pyridinoline; MetraBiosystems Ltd, Wheatley, Oxon., UK) and were measured using a reversed-phase HPLC method with fluorescence detection (Colwell *et al.* 1993). The acetylated pyridinoline was used in accordance with the method described by Calabresi *et al.* (1994) and Robins *et al.* (1994). The crosslinks contents of the urine samples were quantified by external standardisation using a commercially available pyridinoline–deoxypyridinoline HPLC calibrator (MetraBiosystems Ltd). The intra-assay CV for pyridinoline and deoxypyridinoline, measured as the variation between ten chromatograms obtained between column regenerations as described by Colwell *et al.* (1993), were 6 and 9% respectively. The inter-assay CV for pyridinoline and deoxypyridinoline were 7 and 8% respectively.

Femoral mass, length, volume and density. The length of each right femur was measured with a vernier caliper. Bone volume and density were measured by Archimedes' principle (Kalu *et al.* 1991). Briefly, each bone was cut at the mid-diaphysis and the marrow was washed out. Each bone was then put in an unstoppered vial filled with distilled water, and the vial was placed under a vacuum for 90 min to ensure that all the trapped air diffused out of the bone. Each bone was removed from its vial, blotted with gauze, weighed and returned to the vial containing distilled water. The bone was re-weighed in a boat suspended, but completely immersed, in water previously equilibrated to room temperature, and the density (weight/bone volume) was calculated. Bone mass was expressed in terms of the dry weight and bone density as the dry weight per unit volume. Bone mineral mass was expressed as the ash weight per length of bone.

Phosphorus, calcium and magnesium levels of femora and demineralised bone powder. Weighed femora and samples of demineralised bone powder (dried overnight at 110°C) were dry ashed at 600°C for 16 h as described by Hoshino *et al.* (1998) and the ash content was calculated by weight loss on a dry basis. The ash was digested with 16 M-HNO₃. Ca and Mg were analysed in duplicate in femoral and demineralised bone powder digests by atomic absorption spectrophotometry (model SP9; Pye-Unicam, Cambridge, Cambs., UK) after appropriate dilution with LaCl₃ solution (5 g/l; BDH Ltd, Poole, Dorset, UK). A range of Ca and Mg standards were used to obtain Ca and Mg calibration curves. The intra- and inter-assay CV for Ca were 2.8 and 7.8%, and for Mg were 3.2 and 8.8%, respectively. P was determined in the femoral and demineralised bone powder digests in duplicate by the method of Weissman & Pileggi (1974). The intra- and inter-assay CV for P were 4.2 and 6.1% respectively. The accuracy of mineral analysis was assured in each analytical run by appropriate recovery of mineral in dry-ashed samples of bone meal certified by the National Institute of Standards and Technology (standard reference material no. 1486; Laboratory of the Government Chemist, London, UK). Recoveries of Ca, Mg and P in the standard reference material (99.4, 97.5 and 99.2% respectively) were within the tolerance limits.

Femoral epiphyses and bone implant alkaline phosphatase and acid phosphatase. Left femoral distal epiphyses and one of the bone implants were weighed and then homogenised (Janke & Kunkel IKA[®] Labortechnik Ultra-Turrax T25; AGB Scientific Ltd, Dublin, Republic of Ireland) in 0.15 M-NaCl containing 3 mM-NaHCO₃ using two 15 s bursts with a 10 s rest at the maximum setting. The homogenate was centrifuged for 15 min at 4500 g at 4°C. The saline-soluble supernatant fraction was used in the assay for determining alkaline and acid phosphatase activities spectrophotometrically using commercially available kits (Sigma Chemical Co. Ltd, Poole, Dorset, UK). The manufacturer's protocols (procedure no. 104, revised September 1997) were followed for each assay. One Sigma unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol *p*-nitrophenol from *p*-nitrophenyl phosphate (Sigma Chemical Co. Ltd) per h at 37°C. One Sigma unit of phosphatase activity/ml could be converted into IU/l by multiplying by 16.7 (Sigma Chemical Co. Ltd). Protein was determined by using the method of Lowry *et al.* (1951) and the enzyme activities were expressed as IU/mg protein.

Serum osteocalcin levels. Serum osteocalcin concentrations were measured in duplicate using a recently developed ELISA (Biomedical Technologies Inc., Stoughton, MA, USA). The intra- and inter-assay CV were 4.0 and 6.2% respectively.

Reverse transcription–polymerase chain reaction analysis for bone implant insulin-like growth factor-1 mRNA. RNA was isolated and analysed in a bone implant from each rat within a group (*n* 7) as described previously (Fleet & Hock, 1994). Total RNA (1 µg) from each rat was made into cDNA by a reverse transcription reaction as described by Fleet & Hock (1994). The cDNA solution (containing 0.1 µg RNA equivalent) was

then amplified by polymerase chain reaction for forty cycles for both IGF-I and glyceraldehyde phosphate dehydrogenase (used as a constitutively expressed control gene), as described by Fleet & Hock (1994). To minimise the potential for variability in the reverse transcriptase reaction, cDNA was prepared from total cellular RNA for all samples at the same time, using the same reagents. A polymerase chain reaction blank consisting of polymerase chain reaction cocktail and water in place of the RNA sample was included during each amplification; under no circumstance did the polymerase chain reaction product bands appear in these control samples. Polymerase chain reaction products were subjected to agarose (20 g/l) gel electrophoresis. Relative amounts of amplified polymerase chain reaction product from each experimental condition were visualised under u.v. light and digitised with the Kodak Digital Science DC 120 Zoom digital camera and electrophoresis documentation system (Eastman Kodak Company, Rochester, NY, USA). Relative amounts of the product were estimated by digital densitometry using Kodak Digital Science Analysis System 120 quantification software (Eastman Kodak Company). IGF-I expression was normalised relative to the expression of glyceraldehyde phosphate dehydrogenase mRNA.

Statistical methods

Prior to the start of the experiment, the required sample size at α 0.05 and β 0.80 was calculated (Dallal, 1990) using the variability of the mean alkaline phosphatase levels in bone implant and femoral epiphyses of young rats and a selected minimum detectable percentage difference (i.e. Δ) in alkaline phosphatase levels among groups of 165%. An increase in bone alkaline phosphatase activity of 165% has been shown to correlate with increased ^{45}Ca uptake into bone (Sinha *et al.* 1988b), and therefore was considered a meaningful biological difference. Data for all variables were normally distributed and allowed for parametric tests of significance. Results are presented as mean values with their standard errors. All data were subjected to one-way ANOVA, with variation attributed to dietary WP content (Snedecor & Cochran, 1967). Where significant ($P < 0.05$) differences were found, Fisher's least significant difference test was used to perform *post hoc* comparison of all pairs of means (Snedecor & Cochran, 1967).

Results

Mean body-weight gain did not differ among groups at any stage (Fig. 1). Physical properties (length, dry weight, volume, ash, density) and macromineral content (Ca, Mg, P, bone mineral mass) of femora were unaffected by dietary WP content (Table 2). There was no significant effect of dietary WP on serum osteocalcin or urinary pyridinoline and deoxypyridinoline levels (Table 3).

Fourteen days after implantation, the harvested subcutaneous implants of demineralised bone powder appeared as pink plaque-like structures that were approximately

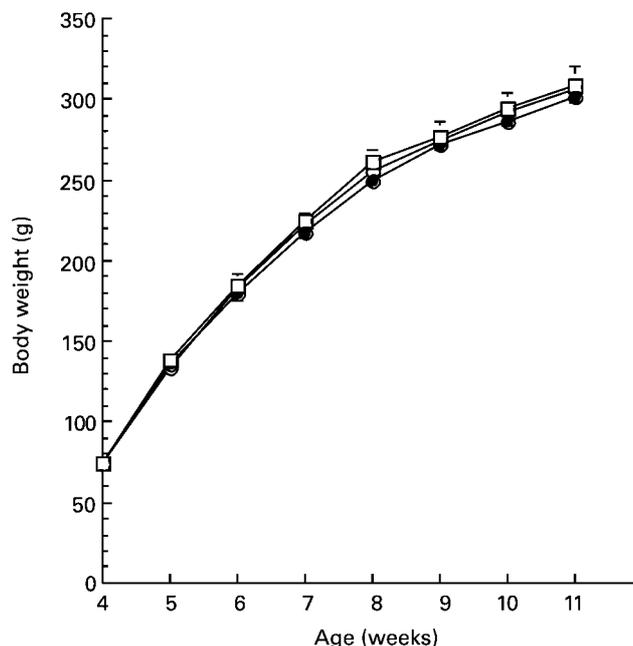


Fig. 1. Change in body weight with time in rats fed on diets containing different levels of bovine whey protein (WP). \square , Control diet; \bullet , WP1 diet (10 g WP/kg); \circ , WP2 diet (20 g WP/kg). For details of diets and procedures, see Table 1 and p. 558. Values are means with their standard errors represented by vertical bars (seven rats per group). No significant differences were found between groups at any of the time points (ANOVA, $P < 0.05$).

10 mm in diameter. There were no macroscopic differences in the bone implants from the different treatment groups.

The influence of dietary WP content on alkaline and acid phosphatase activities in femoral epiphyses and bone implants in young growing rats is shown in Table 4. Neither acid nor alkaline phosphatase activity in femoral epiphyses was affected by dietary WP content. In the bone implants, on the other hand, while acid phosphatase activity was unaffected, alkaline phosphatase activity was significantly ($P < 0.01$) increased by increasing the dietary WP concentration of the diet from 0 to 10 g/kg diet (in place of dietary casein). However, increasing the WP content further to 20 g/kg diet had no further effect on alkaline phosphatase activities.

Bone implant IGF-I mRNA levels were significantly ($P < 0.05$) increased by increasing the dietary WP concentration of the diet from 0 to 10 g/kg diet (in place of dietary casein) (Fig. 2). However, increasing the WP content further to 20 g/kg diet had no further effect on IGF-I mRNA levels (Fig. 2).

Discussion

In the present study, increasing the content of bovine WP in the low-Ca diet for 7 weeks had no effect on bone mass or bone mineral composition in young growing rats. Takada *et al.* (1997a,c) reported that orally administered bovine WP (10 and 20 g/kg diet; the same concentrations as used in the present study) or WP fractions (10 g/kg diet) for 4 weeks had no effect on bone mineral content in ovariectomised rats that had been previously fed on a

Table 2. Bone physical properties and mineral analysis of right femurs in young male rats fed different levels of dietary whey protein* (Mean values with their standard errors for seven rats per group)

Dietary treatment...	AIN-93G		WP1†		WP2†		Statistical significance of effect (one-way ANOVA, <i>P</i>)
	Mean	SE	Mean	SE	Mean	SE	
Femur:							
Length (mm)	31.80	0.34	31.83	0.20	31.62	0.26	0.849
Dry wt (mg)	394	10	402	10	395	10	0.754
Ash wt (mg)	240	3	242	9	241	4	0.852
Ash (% based on dry wt)	60.8	0.5	60.7	0.8	61.1	0.5	0.886
Volume (mm ³)	440	30	440	10	440	20	0.995
Density (g/mm ³)	0.00130	0.00001	0.00132	0.00001	0.00129	0.00003	0.725
Bone mineral mass (mg/mm)	7.54	0.11	7.68	0.26	7.63	0.10	0.833
Ca (mg/g dry wt)	281	5	281	15	275	16	0.934
Mg (mg/g dry wt)	4.60	0.24	4.69	0.32	5.13	0.47	0.536
P (mg/g dry wt)	129	3	128	5	132	9	0.906

WP, whey protein.

* For details of diets and procedures, see Table 1 and p. 558.

† WP1, 10 g WP/kg diet; WP2, 20 g WP/kg diet.

low-Ca (3.0 g/kg) diet for 4 weeks. However, despite a lack of effect on bone mineral content, Takada *et al.* (1997a,c) found that increasing the dietary content of WP or WP fractions was effective in increasing the breaking strength and breaking energy of bone and the total amino acid content in bone (and especially that of collagen-specific amino acids, such as proline and hydroxyproline) in these ovariectomised rats. Unfortunately, we were not in a position to determine the effect of WP on bone strength in the young growing rats in the present study. The exact mechanism by which dietary WP increased bone strength in the studies by Takada *et al.* (1997a,c) is unclear, but it is likely to be as a consequence, at least in part, of an alteration in bone metabolism.

In the present study, increasing the content of WP in the diet had no effect on anatomic whole-body bone formation or bone resorption (as measured by urine- and serum-based sensitive and specific biochemical markers) in young growing rats. Furthermore, increasing the dietary WP had no effect on femoral epiphyseal alkaline and acid phosphatase activities. While these findings might suggest that dietary WP did not alter bone metabolism in these young growing rats, the animals were fed their respective diets for only 7 weeks and this may not have been sufficient time for measurable changes in either total femoral or epiphyseal bone that might arise from the increased dietary WP level. For

example, Sinha *et al.* (1988a) have suggested that studies of nutrition and bone integrity are difficult to perform because of the highly stable nature of bone, which makes the extremely small changes that occur very hard to detect. In addition, the detection of such changes requires extended periods of time, usually many months in the case of laboratory animals (Sinha *et al.* 1988a). As an alternative approach, Sinha *et al.* (1988a,b) have described two short-term *in vivo* bone metabolism models that can be used to assess the impact of nutrients on bone metabolism rapidly, i.e. subcutaneous implants of demineralised and mineralised bone powder for assessing bone formation and resorption respectively. The model of ectopic bone formation, in particular, has been used in studies of the effect of nutrients, such as vitamin A (DeSimone & Reddi, 1983), vitamin D (Sinha *et al.* 1988a; Fleet *et al.* 1996), Mg (Schwartz & Reddi 1979), Ca (Sinha *et al.* 1988a,b), high dietary protein (lactalbumin, Funaba *et al.* 1990; casein, Weiss *et al.* 1981) and casein phosphopeptides (Matsui *et al.* 1994), on the processes of bone formation, as determined by biochemical markers, bone histology and ⁴⁵Ca uptake assays.

In the present study, increasing the dietary WP content significantly increased the activity of alkaline phosphatase in (demineralised) bone implants, suggesting an effect on osteoblastic activity. Sinha *et al.* (1988b) reported that when experimental animals were fed for a total of 5 weeks,

Table 3. Serum osteocalcin and urinary pyridinoline (Pyr) and deoxypyridinoline (Dpyr) levels in young male rats fed different levels of dietary whey protein* (Mean values with their standard errors for seven rats per group)

Dietary treatment...	AIN-93G		WP1†		WP2†		Statistical significance of effect (one-way ANOVA, <i>P</i>)
	Mean	SE	Mean	SE	Mean	SE	
Serum osteocalcin (μg/l)	70.8	11.1	70.1	5.0	64.1	7.8	0.824
Urinary Pyr (nmol/d)	4.3	0.7	5.2	0.7	4.9	0.8	0.720
Urinary Dpyr (nmol/d)	5.0	0.6	5.9	0.5	5.2	0.3	0.491

WP, whey protein.

* For details of diets and procedures, see Table 1 and p. 558.

† WP1, 10 g WP/kg diet; WP2, 20 g WP/kg diet.

Table 4. Alkaline and acid phosphatase levels of left femoral epiphyses and bone implants in young male rats fed different levels of dietary whey protein*

(Mean values with their standard errors for seven rats per group)

Dietary treatment . . .	AIN-93G		WP1†		WP2†		Statistical significance of effect (one-way ANOVA, <i>P</i>)
	Mean	SE	Mean	SE	Mean	SE	
Femoral epiphyseal:							
Acid phosphatase (IU/mg protein)	23.9 ^a	2.4	22.9 ^a	2.2	23.6 ^a	2.9	0.288
Alkaline phosphatase (IU/mg protein)	31.7 ^a	5.7	28.9 ^a	4.6	29.1 ^a	2.5	0.896
Bone implant:							
Acid phosphatase (IU/mg protein)	29.8 ^a	1.8	32.1 ^a	2.5	32.4 ^a	1.6	0.481
Alkaline phosphatase (IU/mg protein)	15.0 ^a	1.3	27.9 ^b	3.1	27.1 ^b	3.0	0.005

WP, whey protein.

^{a,b}Mean values within a row with unlike superscript letters were significantly different (ANOVA followed by Fisher's least significant difference test, $P < 0.01$).

*For details of diets and procedures, see Table 1 and p. 558.

†WP1, 10 g WP/kg diet; WP2, 20 g WP/kg diet.

the changes in the implants (alkaline phosphatase, ⁴⁵Ca uptake and Ca content) were not reflected in the results obtained from the femoral epiphyses, suggesting the implant model was a more rapidly responding bone model. Furthermore, Sinha *et al.* (1988b) showed that similar changes in ectopic bone implants and femoral epiphyses for alkaline phosphatase, ⁴⁵Ca uptake and Ca content could be achieved by dietary manipulation (i.e. altering dietary Ca concentrations) for an extended period of time. The magnitude of the WP-induced increment in bone implant alkaline phosphatase activity (i.e. approximate doubling) observed in the present study has been shown to be associated with increased

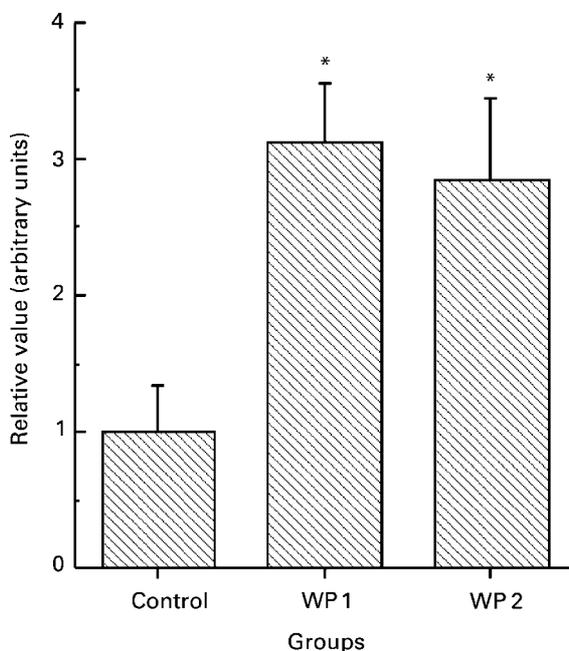


Fig. 2. Effect of bovine whey protein (WP) supplementation on insulin-like growth factor-I mRNA levels in 14 d old subcutaneous bone implants from young growing rats. Control, low-Ca basal (no WP) diet; WP1, 10 g WP/kg; WP2, 20 g WP/kg. For details of diets and procedures, see Table 1 and p. 558. Values are means with their standard errors represented by vertical bars (seven bone implants per group). The effect of WP on mRNA levels was analysed using one-way ANOVA followed by Fisher's least significant difference test. Mean value was significantly different from that of the control: * $P < 0.05$.

⁴⁵Ca uptake into rat bone (Sinha *et al.* 1988b), reflective of increased bone calcification. WP has been shown to stimulate the proliferation and differentiation of murine osteoblastic MC3T3-E1 cells *in vitro*, and to increase the total protein and hydroxyproline contents in these cells (Takada *et al.* 1996). Interestingly, in the study by Takada *et al.* (1996), the stimulatory effect on osteoblast proliferation and differentiation observed with undigested WP was also evident with WP that had been subjected to a proteinase digestion, using a rat everted gut-sac. Takada *et al.* (1997c) suggested that the active components in WP are important in bone formation due to activation of osteoblasts. The findings of the present study, which show a WP-induced increase in osteoblastic activity, would support this contention. Furthermore, the effect of a WP fraction on bone formation has recently been investigated in two dietary intervention studies in human subjects (Aoe *et al.* 2001; Toba *et al.* 2001). While milk basic protein (the basic fraction of WP) increased the rate of bone formation (as determined by serum osteocalcin levels) in healthy adult men (Toba *et al.* 2001), it had no effect on the rate of bone formation in healthy adult women (Aoe *et al.* 2001). It should be noted, however, that the men in the former study received 300 mg milk basic protein/d for 16 d, whereas the women in the latter study only received 40 mg/d for 6 months.

The mechanism by which dietary WP stimulates osteoblastic cell activity is unclear. In the present study, increasing the dietary WP concentration led to an up-regulation of IGF-I gene transcription in the ectopic bone implants; assuming that this increased transcription was translated into increased synthesis of the protein, although this was not determined in the present study, this may, at least in part, account for the changes described in the circulating levels of the protein in human subjects supplemented with bovine milk protein. For example, Schurch *et al.* (1998) reported that milk protein supplementation of elderly patients with femoral fracture attenuated bone loss within the first year post-fracture, which may have been associated with the increased serum IGF-I concentrations observed in these patients (Porter & Johnson, 1998). The increased IGF-I expression in growing bone tissue of rats supplemented with WP in the present study may have led to the observed stimulatory effect on osteoblastic activity in the ectopic bone. IGF-I enhances chondrocyte

proliferation in the growth plate; in bone tissue, especially growing bone, it stimulates osteoblast proliferation and differentiation and matrix formation, including the synthesis of type I collagen and other protein components (Price *et al.* 1994). Interestingly, in addition to an effect of IGF-I on longitudinal bone growth, it can also influence the widening of cortical bone during growth (Yakar *et al.* 2002); this arises from osteoblastic bone formation on the outer bone surface, the periosteum. Therefore, IGF-I plays an important role in the acquisition of bone mass and bone strength. A WP-induced increase in bone IGF-I levels as seen in the present study may have a role, at least in part, in the increased bone strength observed in ovariectomised rats supplemented with dietary WP (Takada *et al.* 1997a,c).

The identity of the active component of dietary WP that brought about the changes in osteoblastic cell activity in the ectopic bone in the present study is unclear. Takada *et al.* (1996) suggested that the stimulatory effect of WP on proliferation and differentiation of the osteoblastic MC3T3-E1 cells may be linked to IGF-I and -II and epidermal growth factor, which are also mitogenic, polypeptide growth factors isolated from WP (Ballard *et al.* 1982; Francis *et al.* 1988; Cox & Bürk, 1991). For example, they found that neutralising IGF-I and -II and epidermal growth factor with the corresponding antibodies negated the stimulatory effects of WP on cell proliferation of the osteoblastic MC3T3-E1 cells. While IGF-I is subjected to proteolytic degradation in the intestine *in vivo*, there is evidence that some IGF-I can be transported intact across the intestine in sucking (Phillips *et al.* 2002) and adult rats (Kimura *et al.* 1997). However, the absorbed IGF-I only appeared in portal and not in peripheral blood (Phillips *et al.* 2000), suggesting that exogenously derived IGF-I would not have biological activity at the level of bone. Interestingly, Ammann *et al.* (2002) recently reported that supplementation of a low-protein diet with essential amino acids led to increased bone strength, reduced bone loss, and increased and reduced rates of bone formation and resorption in ovariectomised rats, possibly by an IGF-I-mediated process. These findings raise the possibility that it may have been essential amino acids derived from the supplemental WP that activated the osteoblasts in the present study. On the other hand, Yamamura *et al.* (1999) proposed that the active component of WP, which is absorbable across the intestinal epithelium and resides in basic fraction of WP (Takada *et al.* 1996), is a high-mobility group protein 1-like protein. High-mobility group protein 1 is well known as a nuclear non-histone chromosomal protein implicated in DNA replication and cellular differentiation (Yamamura *et al.* 1999).

In the present study, acid phosphatase activity in the ectopic bone implants was unaffected by WP. However, the ectopic demineralised bone powder model used in the present study is not appropriate to test the effect of WP on the rate of bone resorption, but rather an ectopic mineralised bone powder model would need to be employed. Several studies have reported a suppressive effect of WP or its basic fraction (milk basic protein) on bone resorption *in vitro* in osteoclasts in culture (Takada *et al.* 1997b) and *in vivo* in ovariectomised rats (Toba *et al.* 2000) and in healthy adult men (Toba *et al.* 2001) and women (Aoe

et al. 2001). The effect of WP on ectopic bone resorption, especially during a period of rapid bone growth, remains to be investigated.

In conclusion, the findings of the present study in a well-established rapid response *in vivo* model of ectopic bone formation suggest that bovine WP can enhance the rate of bone formation in young growing rats fed a Ca-restricted diet. This effect may be mediated by an increased synthesis of IGF-I in growing bone. However, as with most model systems, the ectopic model used in the present study has limitations, including uncertainty as to whether a subcutaneous environment is the same as that found in the skeleton, and the fact that local growth factors may vary in type as well as concentration (Sinha *et al.* 1988a). Therefore, the effect of WP on bone formation and IGF-I expression would need to be confirmed in intact skeletal bone of young growing animals in a feeding trial lasting several months. In addition, identification of the component(s) in WP that can activate osteoblastic cell activity requires further investigation.

References

- Ammann P, Laib A, Bonjour JP, Meyer JM, Rueggsegger P & Rizzoli R (2002) Dietary essential amino acid supplements increase bone strength by influencing bone mass and bone microarchitecture in ovariectomized adult rats fed an isocaloric low-protein diet. *J Bone Miner Res* **17**, 1264–1272.
- Aoe S, Toba Y, Yamamura J, *et al.* (2001) Controlled trial of the effects of milk basic protein (MBP) supplementation on bone metabolism in healthy adult women. *Biosci Biotechnol Biochem* **65**, 913–918.
- Ballard FJ, Nield MK, Francis GL, Dahlenburg GW & Wallace JC (1982) The relationship between the insulin content and inhibitory effects of bovine colostrum on protein breakdown in cultured cells. *J Cell Physiol* **110**, 249–254.
- Calabresi E, Lasagni L, Franceschelli F, Bartolini L & Serio M (1994) Use of an internal standard to measure pyridinoline and deoxypyridinoline in urine (letter). *Clin Chem* **40**, 336–337.
- Colwell R, Russell RGG & Eastell R (1993) Factors affecting the assay of urinary 3-hydroxypyridinium cross-links of collagen as markers of bone resorption. *Eur J Clin Invest* **23**, 341–349.
- Cox DA & Bürk RR (1991) Isolation and characterisation of milk growth factor, a transforming-growth-factor-beta 2-related polypeptide, from bovine milk. *Eur J Biochem* **197**, 353–358.
- Dallal GE (1990) PC-Size consultant – A program for sample size determinations. *Am Stat* **44**, 243.
- DeSimone DP & Reddi AH (1983) Influence of vitamin A on matrix-induced endochondral bone formation. *Calcif Tissue Int* **35**, 732–739.
- Fleet JC, Cashman K, Cox K & Rosen V (1996) The effects of aging on the bone inductive activity of recombinant human bone morphogenetic protein-2. *Endocrinology* **137**, 4605–4610.
- Fleet JC & Hock JM (1994) Identification of osteocalcin mRNA in nonosteoid tissue of rats and humans by reverse transcription–polymerase chain reaction. *J Bone Miner Res* **9**, 1565–1573.
- Francis GL, Upton FM, Ballard FJ, McNeil KA & Wallace JC (1988) Insulin-like growth factors 1 and 2 in bovine colostrum. Sequences and biological activities compared with those of a potent truncated form. *Biochem J* **251**, 95–103.
- Funaba M, Kawashima T, Yano H & Kawashima R (1990) Effects of a high protein diet on bone formation and calcium metabolism in rats. *J Nutr Sci Vitaminol* **36**, 559–567.

- Hoshino H, Kushida K, Takahashi M, Koyama S, Yamauchi H & Inoue T (1998) Effects of low phosphate intake on bone and mineral metabolism in rats: evaluation by biochemical markers and pyridinium cross-link formation in bone. *Ann Nutr Metab* **42**, 110–118.
- Ito T (1991) Science of breast milk. *New Food Industry* **33**, 73–80.
- Kalu DN, Liu CC, Salerno E, Hollis B, Echon R & Ray M (1991) Skeletal response of ovariectomized rats to low and high doses of 17 beta-estradiol. *Bone Miner* **14**, 175–187.
- Kimura T, Murakawa Y, Ohno M, Ohtani S & Higaki K (1997) Gastrointestinal absorption of recombinant human insulin-like growth factor-I in rats. *J Pharmacol Exp Ther* **283**, 611–618.
- Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Matsui T, Yano H, Awano T, Harumoto T & Saito Y (1994) The influences of casein phosphopeptides on metabolism of ectopic bone induced by decalcified bone matrix implantation in rats. *J Nutr Sci Vitaminol* **40**, 137–145.
- Philipps AF, Dvorak B, Kling PJ, Grille JG & Koldovsky O (2000) Absorption of milk-borne insulin-like growth factor-I into portal blood of suckling rats. *J Pediatr Gastroenterol Nutr* **31**, 128–135.
- Philipps AF, Kling PJ, Grille JG & Dvorak B (2002) Intestinal transport of insulin-like growth factor-I (IGFI) in the suckling rat. *J Pediatr Gastroenterol Nutr* **35**, 539–544.
- Porter KH & Johnson MA (1998) Dietary protein supplementation and recovery from femoral fracture. *Nutr Rev* **56**, 337–340.
- Price JS, Oyajobi BO & Russell RG (1994) The cell biology of bone growth. *Eur J Clin Nutr* **48**, S131–S149.
- Reeves PG, Nielsen FH & Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.
- Robins SP, Stead DA & Duncan A (1994) Precautions in using an internal standard to measure pyridinoline and deoxypyridinoline in urine (letter). *Clin Chem* **40**, 2322–2323.
- Rodan GA & Rodan SB (1995) The cells of bone. In *Osteoporosis: Etiology, Diagnosis, and Management*, 2nd ed. pp. 1–39 [BL Riggs and LJ Melton III, editors]. Philadelphia, PA: Lippincott-Raven.
- Schurch MA, Rizzoli R, Slosman D, Vadas L, Vergnaud P & Bonjour JP (1998) Protein supplements increase serum insulin-like growth factor-I levels and attenuate proximal femur bone loss in patients with recent hip fracture. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **128**, 801–809.
- Schwartz R & Reddi AH (1979) Influence of magnesium depletion on matrix-induced endochondral bone formation. *Calcif Tissue Int* **29**, 15–20.
- Sinha R, Smith JC Jr & Soares JH Jr (1988a) Calcium and vitamin D in bone metabolism: analyses of their effects with a short-term in vivo bone model in rats. *J Nutr* **118**, 99–106.
- Sinha R, Smith JC & Soares JH (1988b) The effect of dietary calcium on bone metabolism in young and aged female rats using a short-term in vivo model. *J Nutr* **118**, 1217–1222.
- Snedecor GW & Cochran WG (1967) *Statistical Methods*. Ames, IA: Iowa State University Press.
- Takada Y, Aoe S & Kumegawa M (1996) Whey protein stimulated the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun* **223**, 445–449.
- Takada Y, Kobayashi N, Kato K, Matsuyama H, Yahiro M & Aoe S (1997a) Effects of whey protein on calcium and bone metabolism in ovariectomized rats. *J Nutr Sci Vitaminol* **43**, 199–210.
- Takada Y, Kobayashi N, Matsuyama H, *et al.* (1997b) Whey protein suppresses the osteoclast-mediated bone resorption and osteoclast cell formation. *Int Dairy J* **7**, 821–825.
- Takada Y, Matsuyama H, Kato K, *et al.* (1997c) Milk whey protein enhances the bone breaking force in ovariectomized rats. *Nutr Res* **17**, 1709–1720.
- Toba Y, Takada Y, Matsuoka Y, *et al.* (2001) Milk basic protein promotes bone formation and suppresses bone resorption in healthy adult men. *Biosci Biotechnol Biochem* **65**, 1353–1357.
- Toba Y, Takada Y, Yamamura J, *et al.* (2000) Milk basic protein: a novel protective function of milk against osteoporosis. *Bone* **27**, 403–408.
- Weiss RE, Gorn A, Dux S & Nimni ME (1981) Influence of high protein diets on cartilage and bone formation in rats. *J Nutr* **111**, 804–816.
- Weissman N & Pileggi VJ (1974) Inorganic ions. In *Clinical Chemistry: Principles and Techniques*, pp. 639–755 [RJ Henry, DC Cannon and JW Winkelman, editors]. Hagerstown, MD: Harper and Row.
- Yakar S, Rosen CJ, Beamer WG, *et al.* (2002) Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest* **110**, 771–781.
- Yamamura J, Takada Y, Goto M, Kumegawa M & Aoe S (1999) High mobility group-like protein in bovine milk stimulates the proliferation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun* **261**, 113–117.