Age-related differences in chemical composition of rat femur as determinants for strain

Kari Indrekvam[1], Inger Vikøyr[1], Rolf Terje Lie[2], Lars B. Engesæter[1] and Norvald Langeland[1]

We have explored previous findings of remarkably stable in vivo strain at the femoral surface in rats at different ages, and at the same time increased bone stiffness. The rate of collagen synthesis (^{14}C-hydroxyproline/total hydroxyproline ratio) decreased with age, whereas mineralization (calcium/hydroxyproline ratio) increased. Smaller amounts of immature collagen, caused by reduced synthesis, and increased mineralization both probably produce a less flexible material. These chemical alterations support the observed increase in structural stiffness and strength with age. Both mineralization and configurations separately seemed to have effects on in vivo strain. However, none of these variables seemed to be the major determinant for strain.

University of Bergen [1]Surgical Research Laboratory, Orthopedic Division, and [2]Section for Medical Informatics and Statistics, Haukeland Hospital, N-5021 Bergen, Norway. Tel +47–5 973040. Fax +47–5 972761
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In vivo strain, or surface deformation, of the femur in running rats seems to be nearly independent of the age of the animal (Biewener et al. 1986, Keller and Spengler 1989, Indrekvam et al. 1991). Both geometric and material characteristics are responsible for the structural properties of bone. It has been shown that mechanical properties measured by conventional in vitro testing are influenced by mineralization (Burstein et al. 1975, Currey 1975). Strain, however, is remarkably stable with age, although somewhat reduced for older rats. We therefore tried to determine the influence of chemical composition and geometry on the strain of rat femur. The contents of collagen, calcium, and phosphorus were measured in femora of rats 6, 12, and 52 weeks of age.

Materials and methods

Twenty-four male Wistar rats (Mol:WIST, Møllegaard Breeding Center, Ejby, Denmark)—three groups of 8 animals—were used. The rats were 6, 12, and 52 weeks old with median body weights of respectively 199 (178–216), 324 (302–334), and 495 (455–530) g. These animals were the same as those of a previous report dealing with in vivo and in vitro recordings of mechanical properties of rat femur (Indrekvam et al. 1991). The results of mechanical and geometric measurements are summarized in Table 1. The animals were given a standard maintenance rat diet (RM1 expanded, Special Diets Services, Witham, England) and water ad libitum.

Collagen, calcium and phosphorus analyses

Excised femora were mechanically cleansed of all soft tissues and hydrolyzed in 6 M hydrochloric acid at 110 °C for 18 hours. The amount of hydroxyproline was used as a measure of the collagen content of bone (Firschein 1969). A sample of 100 µL from each hydrolyzed femur was therefore analyzed for hydroxyproline by an amino acid analyzer (Biotronik Aminoacid Analyzer LC 7000, Wissenschaftliche Garete GMBH, Frankfurt, Germany) with an integrator (Spectra Physics SP 4200 computing integrator, San Jose, CA. U.S.A.).

To measure collagen synthesis, ^{14}C-proline (15 µCi/100 g body weight) was injected intraperitoneally 24 h before killing the rats. The amount of radioactive hydroxyproline in the bone was measured in a beta counter (Wallac 1217 Rackbeta Liquid Scintillation Counter, Turku, Finland) after separation from proline by columns of Dowex 50W X 8. The specific activity, expressing incorporation of collagen during 24 h, was calculated from the ^{14}C-hydroxyproline/total hydroxyproline ratio ad modum Firschein (1969). Calcium and phosphorus analyses were carried out by means of continuous-flow spectrophotometry (Technicon SMAC system, New York, U.S.A.) (Gitelman 1967, Amador and Urban 1972, Daly and Ertinghausen 1972). Mineralization was expressed as the calcium/hydroxyproline ratio.

Table 1. Geometric and mechanical properties of rat femur at different ages. Median values (0.25 and 0.75 fractiles)

Age (weeks)	N	Body weight (g)	Femur length (mm)	Cross-section area (mm^2)	Area moment of inertia (mm^4)	Stiffness (N/mm)	Ultimate load (N)	Strain (μstrain)
6	8	199 (178–216)	30.1 (29.7–30.5)	1.2 (1.1–1.3)	4.3 (4.0–4.7)	202 (183–206)	95 (86–103)	270 (181–347)
12	8	324 (302–334)	34.9 (34.5–35.2)	1.6 (1.5–1.7)	5.5 (5.0–6.5)	434 (357–505)	195 (184–224)	329 (219–392)
52	8	495 (455–530)	40.0 (39.2–40.4)	2.5 (2.3–2.6)	11.9 (10.5–12.4)	715 (600–803)	389 (337–425)	230 (183–256)

Table 2. Hydroxyproline and mineral content in rat femora at 6, 12, and 52 weeks of age. Mineralization (calcium/hydroxyproline ratio) and collagen synthesis (specific activity of ^{14}C-hydroxyproline) for the same age groups. Median (0.25 and 0.75 fractiles)

Age weeks	N	Hydroxyproline (μmol)	Calcium (mmol)	Phosphorus (mmol)	Mineralization (mmol/mmol)	Collagen synthesis (DPM/mmol)
6	8	59.0 (51.8–63.6)	1.7 (1.5–1.8)	1.2 (1.0–1.2)	28.6 (26.9–29.9)	1307 (1218–1409)
12	8	90.7 (85.2–96.4)	2.8 (2.7–3.0)	1.8 (1.7–1.9)	31.4 (30.3–32.6)	537 (458–676)
52	8	133.1 (127.5–149.2)	5.3 (4.8–5.6)	3.2 (2.9–3.4)	40.0 (35.8–42.8)	326 (221–444)

Statistics

Median values with 0.25 and 0.75 fractiles were used to express the average and variance of the measurements. The Kruskal-Wallis test was used to test differences in medians between groups (Minitab, Ryan et al. 1985). Differences were considered significant when $P < 0.05$. The Kendall rank correlation procedure was applied to test for correlation between two variables (SPSS^x, 1976). Factor analysis was used to characterize groups of related variables (BMDP P4M, Frane et al. 1985). Multiple linear regression analysis was used to identify the variables with highest explanation ratio of strain values (BMDP P1R, Dixon 1985).

Results

Total contents of hydroxyproline, calcium, and phosphorus in the femora naturally increased with age ($P < 0.005$). During the initial period, these increased until 12 weeks by factors of 1.5, 1.6, and 1.5, respectively; and during the following 40 weeks, the corresponding figures were 1.5, 1.9, and 1.8 (Table 2). Also the calcium/phosphorus ratio increased during growth ($P < 0.01$). The ratios were 1.46, 1.59, and 1.64 for 6-, 12-, and 52-week-old animals. Mineralization calculated as the calcium/hydroxyproline ratio had higher values in older than in younger bones ($P < 0.005$; Table 2). When the biochemical measurements were compared with mechanical measurements of the same rats, there was a correlation between in vivo stiffness calculated from the strain-gauge recordings (body weight/in vivo strain ratio) and mineralization (calcium/hydroxyproline ratio; Kendall's $tau = 0.74$, $P < 0.001$). The specific activity of ^{14}C-hydroxyproline, representing the rate of collagen synthesis, decreased with age ($P < 0.005$; Table 2).

Statistical factor analysis with two factors revealed that strain seemed to vary independently of the other variables, because strain was the only variable with a high load score on the second factor (Table 3). Linear

Table 3. Factor analysis of age, body weight, geometry, mechanical, and chemical properties of rat femur

	Factor 1	Factor 2
Log age	0.920	–0.363
Body weight	0.949	–0.293
Femur length	0.957	–0.247
Cross-sectional area	0.882	–0.386
Area moment of inertia	0.831	–0.460
Stiffness	0.911	–0.235
Ultimate load	0.917	–0.356
Strain	–0.225	0.952
Mineralization	0.804	–0.498
Specific activity ^{14}C-hydroxyproline	–0.907	0.118
Explained variance	7.32	1.99

Table 4. Linear regression analysis of strain on rat femoral surface by femur length, diaphyseal cross-sectional area, and mineralization

Variable	Coefficient	*P*-value
Intercept	527	
Femur length	8.2	0.422
Cross-sectional area	–45.5	0.595
Mineralization	–12.3	0.105
Multiple R-square (percent)	44.5	

regression analysis indicated that mineralization, cross-sectional area, and femur length separately had an effect on strain ($P = 0.001$, $P = 0.004$, and $P = 0.01$, respectively). However, none of these variables seemed to be the major determinant for strain (Table 4).

Discussion

This study shows that mineralization increased with age, whereas the rate of collagen synthesis decreased. These chemical changes correspond to increased stiffness measured by strain gauges in older rats compared with younger ones (Indrekvam et al. 1991). Mineralization, cross-sectional area, and femur length separately had an effect on strain.

In the present study, the total content of minerals increased with age as reported by Ekeland et al. (1983), but a disproportionate rise in calcium content relative to phosphorus occurred ($P < 0.01$). It has been reported that bones of young individuals contain a greater proportion of amorphous calcium phosphate than bones of old rats, in which most calcium appears as crystalline hydroxyapatite (Termine 1972, Eanes et al. 1973, Lees and Davidson 1977). The calcium/phosphorus (Ca/P) ratio for synthetic amorphous calcium phosphate has been reported to be 1.47, and for hydroxyapatite 1.67 (Grynpas et al. 1984). Elliott (1973) proposed that in initial amorphous deposits of minerals there could be a mixture of salts with different Ca/P ratios.

This could explain why the Ca/P ratio in our study was rather low in the youngest animals and then increasing with age. Although Grynpas et al. (1984) claimed that the initial solid mineral phase in bone is not amorphous, their findings indicate an increase in crystallinity or crystal perfection with age, and increased Ca/P ratio of the same range as our results.

The intimate association of minerals with collagen is essential to the formation of hard connective tissues such as bone. The proportion of minerals in bone tissue, expressed as the calcium/hydroxyproline ratio, was found to be greater in older animals than in younger ones ($P < 0.005$). This is in agreement with Urry's (1974) suggestion that binding of calcium to protein in bone tissue is a continuous process that progresses with age. Both increased mineralization and a higher proportion of crystalline hydroxyapatite in the older bones theoretically make the bone tissue of these animals stiffer (Currey 1975, 1988, Lees and Davidson 1977).

There was a correlation between mineralization and stiffness calculated from the strain recordings. Equally loaded bones with a high calcium/hydroxyproline ratio were less deformed than bones with a low ratio (Indrekvam et al. unpublished data). The older animals also had the highest maximum bending stress compared with the younger ones (Indrekvam et al. 1991). Currey (1975, 1988) reported that greater mineralization in specimens of the bovine femur produced greater ultimate tensile strength and a higher modulus of elasticity. Burstein et al. (1975) found that with progressive decalcification ultimate stress decreased. All of these studies therefore emphasize the importance of mineralization for mechanical properties of bone.

Torzilli et al. (1981) reported from a canine bone study that geometry and material contributed equally to structural changes, whereas others have reported that structural properties of rat bones are dependent on the geometry to a greater extent than on the material (Ekeland et al. 1981, Keller et al. 1986).

Linear regression analysis in the present study revealed that apparently neither geometric measurements nor mineralization are the major determinant of strain. However, mineralization and geometric measures both increase with age, which may cause difficulties to decide statistically the individual importance (i.e., a tendency to multicollinearity).

Perhaps strain can best be regarded as a result of coordinated efforts between geometry and material to meet the environmental requirements of bone; or as expressed by Frost (1983), strain may be a determinant for bone architecture. Under this assumption, different functional demands of daily activity cause similar deformation of bone independent of age, but old bones would need stiffer material and greater geometric dimensions to meet the increasing load from body weight and muscle traction. Growth will cause somewhat higher strain values, because the bones never will be fully adapted.

The biochemical analyses in the present study were performed on whole femora. To insure that minor amounts of other collagens (with different hydroxyproline and calcium contents than type I) did not affect

the results, analyses of hydroxyproline and minerals were performed in 4 extra animals in each age group. On the one side whole femora and on the other side the mid-diaphyseal one third of the bones after cleansing the marrow cavity were analyzed. Changes in mineralization (calcium/hydroxyproline ratio) with age was not significantly different when calculated on the basis of the results from whole femora or diaphyseal parts of femora.

In conclusion, the functional demands of physical activity caused strain of the same magnitude of the age groups tested, although strain was somewhat reduced for older rats. Increased mineralization and a lower rate of collagen synthesis contributed to stiffer bone with age. The results are compatible with a tendency of mechanical behavior of bone to be regulated by changes in both geometry and material properties. Nearly constant strain compared with greater alterations of other variables indicate that strain might be a measure of different bone properties being adjusted to each other to meet the changing mechanical demands during growth and aging.

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