Age- and Gender-related Changes in the Distribution of Osteocalcin in the Extracellular Matrix of Normal Male and Female Bone

Possible Involvement of Osteocalcin in Bone Remodeling

Ronald T. Ingram, Yong-Koo Park, Bart L. Clarke, and Lorraine A. Fitzpatrick
Department of Internal Medicine, Division of Endocrinology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905

Abstract

With increasing age, bone undergoes changes in remodeling that ultimately compromise the structural integrity of the skeleton. The presence of osteocalcin in bone matrix may alter bone remodeling by promoting osteoclast activity. Whether age- and/or gender-related differences exist in the distribution of osteocalcin within individual bone remodeling units is not known. In this study, we determined the immunohistochemical distribution of osteocalcin in the extracellular matrix of iliac crest bone biopsies obtained from normal male and female volunteers, 20–80 yr old. Four different distribution patterns of osteocalcin within individual osteons were arbitrarily defined as types I, II, III, or IV. The frequency of appearance of each osteon type was determined as a percent of the total osteons per histologic section. The proportion of osteons that stained homogeneously throughout the concentric lamellae (type I) decreased in females and males with increasing age. The proportion of osteons that lack osteocalcin in the matrix immediately adjacent to Haversian canals (type III) increased in females and males with age. Osteons staining intensely in the matrix adjacent to Haversian canals (type II) increased in females and was unchanged in aging males. Osteons that contained osteocalcin-positive resting lines (type IV) increased in bone obtained from males with increasing age but were unchanged in females. Sections of bone immunostained for osteopontin (SPP-I), osteonectin, and decorin did not reveal multiple patterns or alterations in staining with gender or increasing age. We suggest that the morphology of individual bone remodeling units is heterogeneous and the particular morphologic pattern of osteocalcin distribution changes with age and gender. These results suggest that differences in the distribution of osteocalcin in bone matrix may be responsible, in part, for the altered remodeling of bone associated with gender and aging. (*J. Clin. Invest.* 1994. 93:989–997.) Key words: bone • aging • noncollagenous proteins • osteocalcin • osteopontin

Address correspondence to Lorraine A. Fitzpatrick, 5-164 W. Joseph Building, Mayo Clinic and Mayo Foundation, Rochester, MN 55905. Ronald Ingram's present address is Telios Pharmaceuticals, 4757 Nexus Centre Drive, San Diego, CA 92122. Yong-Koo Park's present address is the Department of Pathology, School of Medicine, Kyung Hee University Hospital, Seoul, 130-702, Korea.

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Introduction

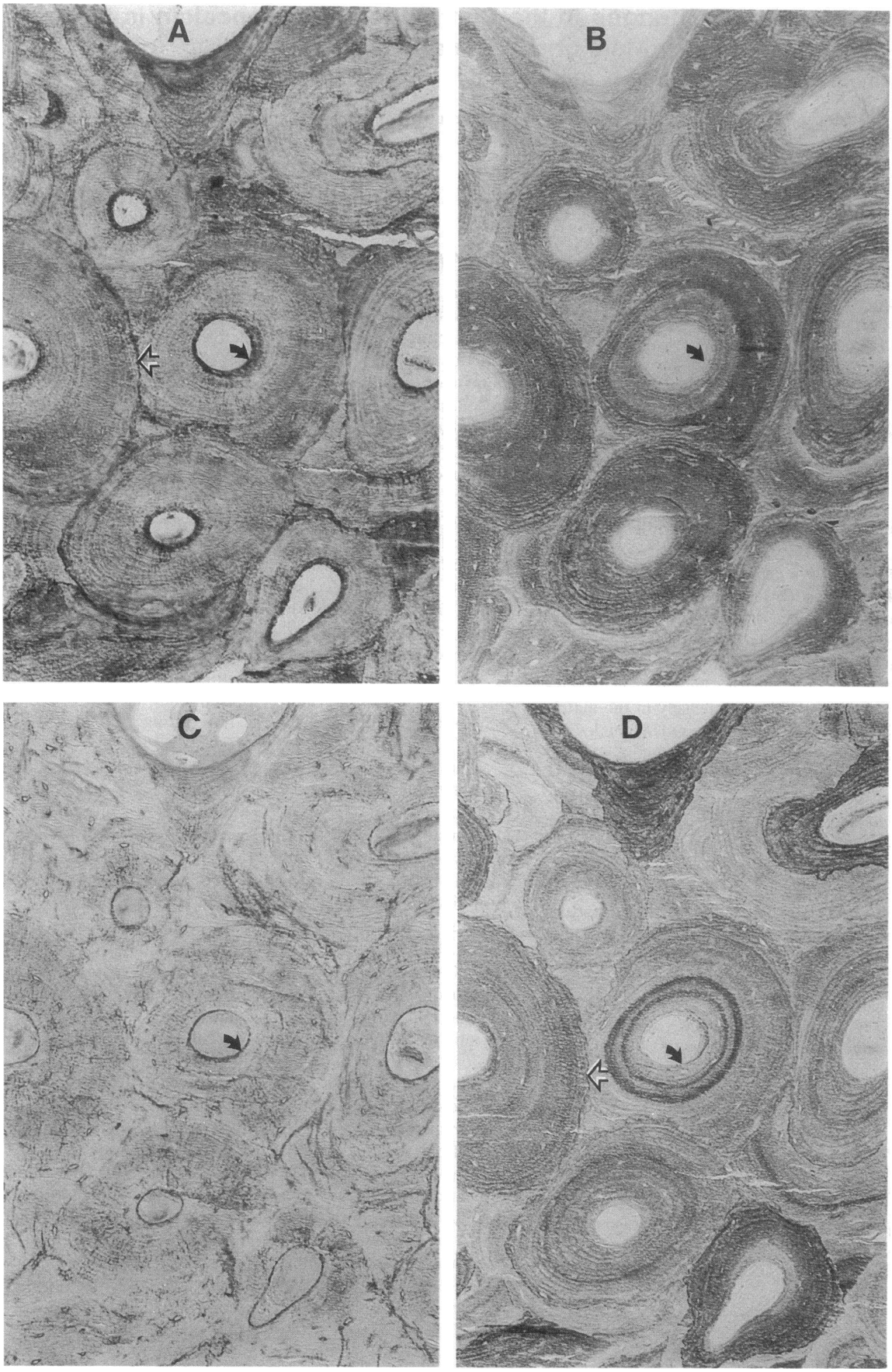
Normal bone metabolism is the result of a highly integrated relationship between bone resorption and formation. With age, the structural integrity of the skeleton declines along with the functional capacity of various organ systems. Although overall bone mass decreases with age in females and males, the rate of bone loss in females is greater than males (1–3). The mechanisms by which bone remodeling is altered with age and gender are poorly understood.

The organic phase of normal bone consists of 90% type I collagen and the remaining 10% is composed of noncollagenous proteins (NCPs).[1] NCPs are synthesized and secreted by bone cells, and their expression is regulated, in part, by local growth factors and hormones (4–7). Accumulating evidence suggests that the extracellular matrix of bone exerts profound effects on cellular activity by retaining NCPs and growth factors that influence both immediate and long-term cell–matrix interactions (8–13). Reduced recruitment of osteoclasts to devitalized particles of bone from human donors of increasing age suggests that bone matrix components may be partially responsible for impaired skeletal remodeling associated with aging (9).

Osteocalcin is a 6.5-kD vitamin K–dependent, gamma-carboxyglutamic acid–containing NCP secreted by osteoblasts and odontoblasts. Due to its high affinity for calcium and hydroxyapatite, osteocalcin is incorporated into the extracellular matrix of bone (14–16). A growing body of evidence suggests that this matrix protein is involved in bone remodeling. Significant differences in the concentration of extractable osteocalcin between cortical and trabecular bone in humans provides evidence of distinct regulatory mechanisms among these areas (10). Osteocalcin-deficient bone particles obtained from warfarin-treated rats recruit fewer osteoclasts and are resorbed less than normal particles using in vivo assays (11, 17, 18). Osteopetrotic rat bone contains low concentrations of osteocalcin, and osteoclast activity in these animals is reduced (19).

The possibility that NCPs are involved in regulating site-specific cell–matrix interactions is supported by studies that have immunolocalized NCPs within the extracellular matrix of bone from several species (13, 20–22). Presently, it is not known whether age or gender differences exist in the microanatomical distribution of NCPs within bone remodeling units. In this study, the immunohistochemical distribution of osteocalcin in the extracellular matrix of bone was examined in iliac crest bone biopsies obtained from normal male and female volunteers, 20–80 yr old. We provide evidence that specific age- and gender-related changes occur in the pattern of distribution of osteocalcin within individual osteons and suggest

1. *Abbreviation used in this paper:* NCP, noncollagenous protein.


A
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C
D

that these biochemical alterations may be responsible, in part, for age- and gender-related differences in bone remodeling.

Methods

Bone biopsies. Iliac crest bone biopsies were obtained under local anesthesia from 41 male and 16 female volunteers (20–80 yr old) after informed consent (Internal Review approval nos. 478-X-90 and 428-B-86). Serum and urine chemical indices were normal, and bone density determined by dual photon absorptiometry or DEXA at the spine and hip were within the 2.5–97.5th percentile for age- and sex-matched controls. Biopsies were fixed in 70% ethanol and dehydrated in ascending ethanols for 4 d at 4°C before embedding.

Embedding. Biopsies were embedded in modified glycol-methylmethacrylate using a temperature-controlled method (Rainier Technical Products, Seattle, WA) as previously described (13). Briefly, biopsies were infiltrated (4 d) in a mixture of the following: 81% (vol/vol) uninhibited methylmethacrylate, 8% (wt/vol) polyethylene glycol distearate (1540), 6.5% (vol/vol) 2-hydroxyethyl methacrylate, 4% dibutylphthalate, and 0.65% benzoyl peroxide. Infiltrated biopsies were placed in a fresh monomer-containing accelerator (JB-4; Polysciences Inc., Warrington, PA) and polymerized onto aluminum chucks at room temperature in the presence of nitrogen. Unmounted sections (5 μm) were immunostained as described below.

Antibodies. Bovine bone–derived proteins were used to generate antisera against osteocalcin (LF-32) and osteonectin (LF-BONII). These antibodies cross-react with their respective human bone–derived proteins (23). Polyclonal antidecorin antibody (LF-30) was generated against human decorin sequence (24). Rabbit polyclonal antiserum for detection of osteopontin (LF-7) was generated against purified human osteopontin (25). Antibodies were kindly provided by Dr. Larry Fisher (National Institute of Dental Research, National Institutes of Health, Bethesda, MD).

Immunohistology. Sections of normal bone were immunostained as described previously (13). Briefly, unmounted sections were rehydrated in 50% ethanol and decalcified with acetic acid (1% for 10 min) followed by blocking in Tris-buffered saline (TBS; 0.05 M Tris, 0.01% BSA, 0.9% NaCl, pH 7.5) containing 0.3% casein (Sigma Chemical Co., St. Louis, MO) and 10% normal goat serum. Sections were stained using VECTASTAIN Elite avidin-biotin complex (ABC) Kit (Vector Laboratories, Burlingame, CA) according to the manufacturers' recommendations, with modifications. All steps were carried out at room temperature and were followed by two 15-min washes in TBS containing 0.02% Triton X-100. Endogenous peroxidase activity was inhibited with 1.5% H_2O_2 and 0.1% sodium azide in 50% methanol for 15 min. Bound antibody was detected with peroxidase-conjugated ABC and visualized using 0.05% diaminobenzidine and 0.01% H_2O_2. Sections were rinsed with tap water, dehydrated with ascending alcohols, cleared with xylene, and mounted on glass slides with coverslips using EUKITT® mounting medium (Calibrated Instruments, Inc., Hawthorne, NY). Control sections were stained using normal rabbit serum at the same dilution as primary antibody. Chondroitinase ABC was used to enhance specific staining of decorin as described elsewhere (26).

Analysis. Depending on the pattern of osteocalcin immunostaining, osteons were arbitrarily defined as types I, II, III, or IV. Patterns of staining were quantified blindly by two observers. The frequency of appearance of each osteon type was determined as a percentage of the total number of osteons per bone section. The correlation coefficients (r) were determined by least squares linear regression or by split-point linear regression, and the P values by Student's two-tailed t test using a statistical analysis system (SAS) computer program. In addition to linear regression, data from male specimens were analyzed by decade of life using ANOVA (n = 6–8/decade). Data from female specimens were divided into premenopausal ($\leq$ 40 yr; n = 7) and postmenopausal groups ($\geq$ 60 yr; n = 9) and analyzed by ANOVA.

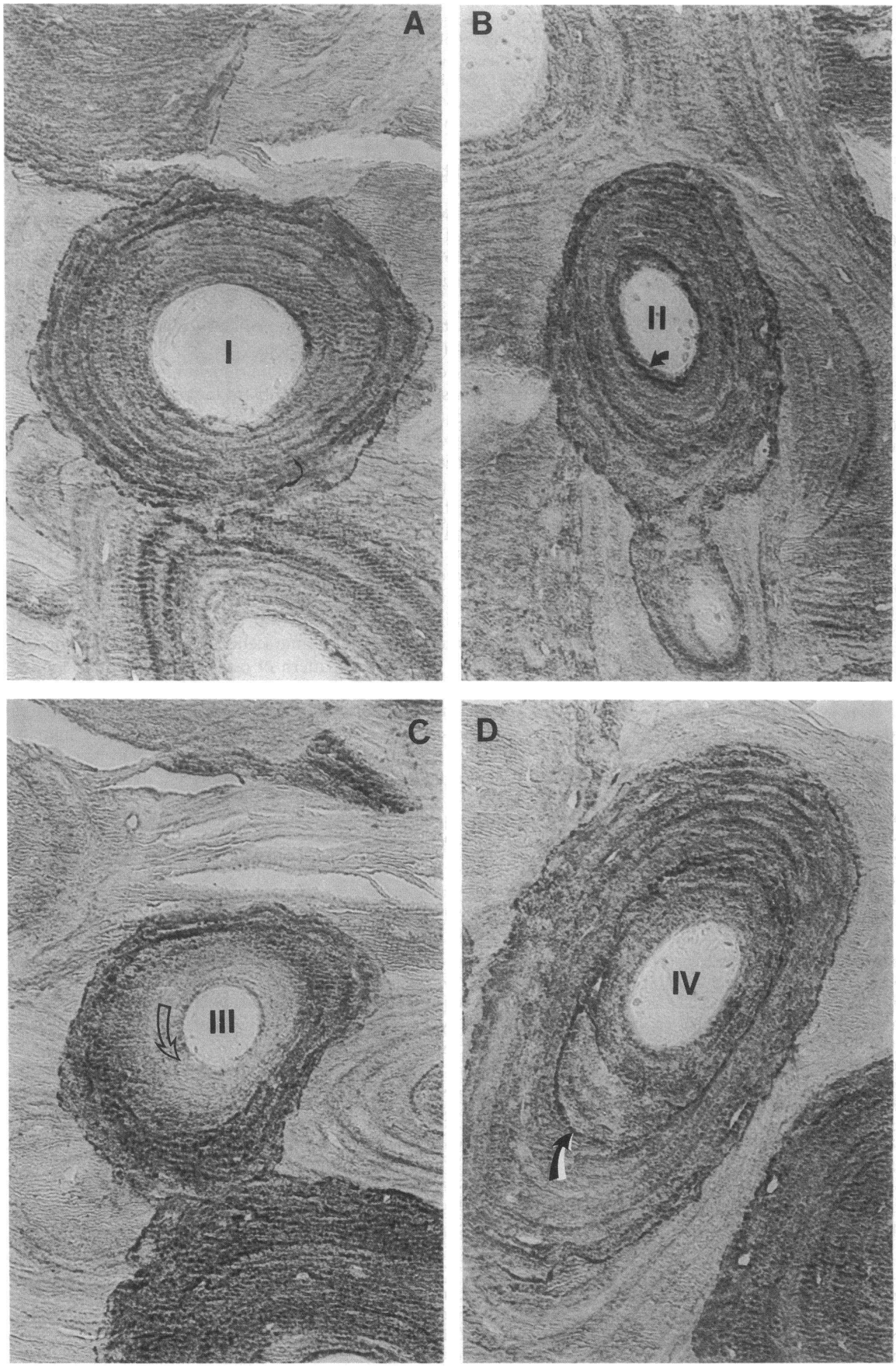
Results

Immunohistochemical staining of NCPs in osteons. The patterns of immunostaining for NCPs were highly reproducible. Both cortical and trabecular bone were positively stained using antiserum to NCPs. Antiserum to osteopontin stained intensely at cement lines of cortical and trabecular (not shown) bone and in the matrix immediately adjacent to Haversian canals (Fig. 1 *A*) as previously described (13). Antiserum against osteonectin did not stain cement lines, and within each osteon staining was weak or absent in the matrix immediately adjacent to Haversian canals (Fig. 1 *B*). A similar pattern of light and dark staining was observed in bone remodeling units of trabecular bone as well (not shown). Decorin was not present in cement lines but was distributed weakly throughout the concentric lamellae and stained intensely in the matrix immediately adjacent to Haversian canals.

The patterns of distribution for osteopontin, osteonectin, and decorin did not change with age or gender. By contrast, the distribution pattern of osteocalcin was heterogeneous both within individual units of bone (osteons) as well as compared to adjacent units on the same section (Fig. 1 *D*). We arbitrarily defined the staining pattern of osteocalcin within osteons as types I, II, III, or IV. Concentric lamellae in type I osteons stained for osteocalcin homogeneously from the cement line to the Haversian canal (Fig. 2 *A*). Type II osteons stained marginally for osteocalcin throughout the lamellae and had intense staining in the matrix immediately adjacent to Haversian canals (Fig. 2 *B*). Type III osteons were defined as those devoid of osteocalcin in the matrix immediately adjacent to Haversian canals (Fig. 2 *C*). Type IV osteons contained a resting line that stained positive for osteocalcin (Fig. 2 *D*). To determine whether the proportion of osteons displaying these patterns of osteocalcin staining changed with age and/or gender, sections of bone were quantified according to the criterion described above.

Frequency of osteon type in aging males. The relationship between osteon types and age in males are shown in Fig. 3, *A–D*. Type I osteons, which stained homogeneously for osteocalcin throughout the osteon (Fig. 2 *A*), were the predominant staining pattern in male bone. The proportion of type I osteons decreased with age (Fig. 3 *A*; $r = -0.64$, $P = 0.0001$) and the range was 69.6±4.0 to 30.3±4.3% (second to seventh decade of life, respectively; Fig. 4). Type II osteons, which stained intensely in the matrix immediately adjacent to Haversian canals (Fig. 2 *B*), did not significantly change with age (Fig. 3 *B*; $r = 0.019$, $P = 0.4$). In contrast to the pattern of distribution of osteocalcin in types I and II osteons, type III osteons lacked

Figure 1. Immunohistochemical staining of osteopontin (*A*), osteonectin (*B*), decorin (*C*), and osteocalcin (*D*) in adjacent sections of normal human cortical bone. Undecalcified bone specimens were fixed in ethanol, dehydrated, and embedded in glycolmethylmethacrylate. Unmounted sections (5 μm) were immunostained after brief decalcification as described in Methods. Cement (reversal) lines stained intensely positive for osteopontin (*A; open arrow*), and osteocalcin (*D; open arrow*) but not for osteonectin (*B*) or decorin (*C*). The matrix immediately adjacent to Haversian canals stained intensely for osteopontin and decorin but not osteonectin or osteocalcin (*filled arrows*). Osteons stained for osteocalcin demonstrated multiple patterns of distribution on the same section of bone. ×100.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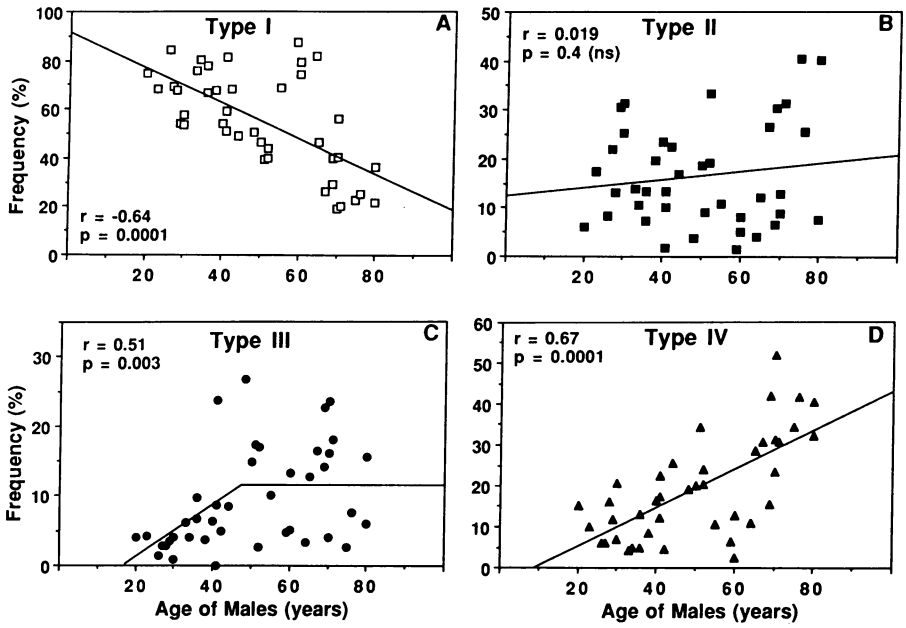

Figure 3. Relationship between the frequency of osteon types I, II, III, or IV and age in bone obtained from male subjects. Sections of iliac crest bone were immunostained for osteocalcin and the proportion (frequency) of osteon types (as a percent of the total number of osteons) in each cortex was quantified. Correlation coefficients (r) were determined by least squares linear regression (*A, B,* and *D*) or by split-point linear regression (*C*), and the P values were obtained by Student's two-tailed t test.

staining in the matrix immediately adjacent to Haversian canals (Fig. 2 *C*). The proportion of type III osteons increased in a nonlinear fashion with age when data were analyzed by split-point linear regression (Fig. 3 *C*; $r = 0.51$, $P = 0.003$). When data were divided by decade of life (Fig. 4), the increase in type III osteons appeared to reach a maximum in the fourth decade of life (11–13%) and remained unchanged through the seventh decade. Type IV osteons were characterized by the presence of a resting line that stained positive for osteocalcin (Fig. 2 *D*). The frequency of type IV osteons increased significantly with age in males (Fig. 3 *D*; $r = 0.67$, $P = 0.0001$). The range was 10.9±1.8 to 35.6±2.9% (Fig. 4; second vs. seventh decade of life, respectively). The average number of osteons per section of bone did not significantly change with age in males or females (data not shown).

Frequency of osteon type in aging females. Type I osteons were predominant in bone obtained from normal females. There was a significant negative correlation between the proportion of type I osteons and increasing age (Fig. 5 *A*; $r = -0.56$, $P = 0.013$) and the range was 68.1±3.4% in premenopausal (≤ 40-yr-old) and 43.3±5.3% in postmenopausal (≥ 60-yr-old) groups (Fig. 6). In contrast, there was a positive correlation with age in the proportion of type II (Fig. 5 *B*; $r = 0.63$, $P = 0.003$) and type III osteons (Fig. 5 *C*; $r = 0.52$, $P = 0.02$). The proportion of types II and III osteons increased two- to threefold between pre- and postmenopausal women (Fig. 6). There was not a significant correlation between the frequency of type IV osteons and age in females (Fig. 5 *D*).

Histomorphometric analysis. Sections of bone were examined by light microscopy to determine if the cellular activity of osteons differed between the four types of osteons. Histologically, there were no significant differences in the cellular activity among osteons of the four defined types. Indices of active formation (number of osteoblasts, osteoid width, osteoid surface, osteoid volume, osteoblast–osteoid interface, or bone formation rates) or resorption (percent eroded surface, number of osteoclasts per surface length) were rarely present within each osteon (data not shown).

Discussion

Osteocalcin represents a late phenotypic marker in the differentiation of osteoblasts (27, 28). Mature osteoblasts secrete osteocalcin into the extracellular space where this protein either enters the circulation or diffuses through osteoid and binds mineralized bone. An important step in the resorption of bone requires the recruitment and fusion of monocytes or macrophages to augment the population of multinucleated osteoclasts (29). Using in vivo bone resorbing assays, several studies suggest that the presence of osteocalcin in the extracellular matrix of bone may represent an important chemotactic signal for resorption. Subcutaneous implants of osteocalcin-deficient bone particles obtained from warfarin-treated rats recruit fewer osteoclast-like cells (11, 17) and resorption of osteocalcin-deficient particles is significantly reduced (18). During bone resorption, degradative products from bone matrix, including osteocalcin, are released into the extracellular space and are thought to further promote monocyte recruitment (30). Accordingly, site-specific differences in osteocalcin in the extracellular matrix may influence site-specific remodeling events.

Although the overall concentration of osteocalcin in bone matrix differs with age and location, demonstration of the microanatomical distribution of osteocalcin in the extracellular matrix of bone has been difficult due to the loss of NCPs during tissue fixation and decalcification (10, 31, 32). In this study, using undecalcified bone specimens embedded in glycol-meth-

Figure 2. Immunohistochemical staining of osteocalcin in normal human cortical bone. Representative staining patterns for osteon types I, II, III, and IV are shown. Type I osteons (*A*) stained homogeneously throughout the concentric lamellae. Type II osteons (*B*) contained intense staining in the matrix immediately adjacent to Haversian canals (*filled arrow*). Type III osteons (*C*) contained weak staining in the matrix immediately adjacent to Haversian canals (*open arrow*). Type IV osteons (*D*) contained resting (arrest) lines (*filled arrow*) that stained intensely positive for osteocalcin. ×200.

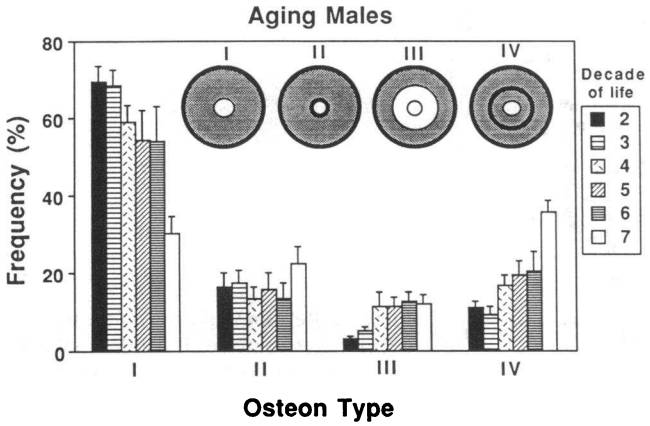

Figure 4. Frequency of osteon types with decade of life in bone obtained from males (n = 6–8/decade). The frequency represents the percentage of the total number of osteons in each section of bone. Values represent the mean frequency±SEM for each decade. The actual number (mean±SEM) of osteons per bone section for each decade of life were as follows: second, 49.1±6.9; third, 56.6±9.8; fourth, 83.4±22.4; fifth, 58.8±7.5; sixth, 57.3±10.7; seventh, 55.8±5.4. Significant differences in the frequency of osteon types were determined by ANOVA and exist in type I (P = 0.0003), type III ($P < 0.05$), and type IV (P = 0.0001) osteons with each decade of life.

ylmethacrylate, we found that the distribution of immunodetectable osteocalcin in osteons was heterogeneous compared to other NCPs such as osteopontin, osteonectin, and decorin. We, therefore, arbitrarily defined the patterns of osteocalcin staining in osteons as types I–IV and determined the proportion of osteons displaying these patterns in specimens obtained from male and female subjects of varying ages. In bone obtained from both female and male subjects, the predominant distribution pattern of osteocalcin in osteons was type I (~ 70%). The concentric lamellae stained homogeneously throughout type I osteons. With advancing age, the proportion of type I osteons decreased and the presence of types II, III, and IV osteons either increased or remained unchanged. The implications of the observed shift in the proportion of osteon types with age may be several-fold.

The proportion of type II osteons increased with age in females and was unchanged in males. Type II osteons were characterized by the presence of intensely stained osteocalcin in the matrix immediately adjacent to Haversian canals. A similar pattern of distribution was observed when sections were stained using antisera against osteopontin. Osteopontin, a phosphorylated glycoprotein, is produced by osteoblasts and incorporated into matrix during bone formation. This NCP, however, differs from osteocalcin in that it belongs to a family of unique phosphorylated glycoproteins that contain the tripeptide sequence Arg-Gly-Asp (RGD) common to cell attachment type proteins that bind integrins (33, 34). Integrins are expressed in osteoblasts and osteoclasts and are requisite for attachment of these cells to bone (34, 35). Attachment, differentiation, and activation of osteoclasts were enhanced in the presence of osteopontin in vitro (12, 36, 37), and osteopontin has been localized to sites of osteoclast attachment in developing rat bone (20). The presence of osteocalcin- and osteopontin-rich matrix may increase the recruitment and binding of osteoclasts. However, given the limited number of osteons containing osteocalcin-rich surfaces compared with those rich in osteopontin (~ 16 vs. 95%, respectively), osteocalcin may represent a site-specific regulator of bone remodeling. In normal postmenopausal females, a two- to threefold increase in the proportion of type II osteons could potentially increase the rate of bone turnover. In contrast, bone specimens from males did not display an age-related change in the proportion of type II osteons, suggesting that this pattern of distribution reflects gender-related differences in bone.

In our study of normal human bone, the proportion of osteons that lack immunodetectable osteocalcin in the matrix adjacent to Haversian canals increased with age in males (Fig. 3 *C*) and females (Fig. 5 *C*). Changes in the proportion of type III osteons with age in males does not appear to be constant. Analysis of data using split-point linear regression (Fig. 3 *C*) and by decade of life (Fig. 4) indicates that the proportion of type III osteons does not change after the fourth decade of life.

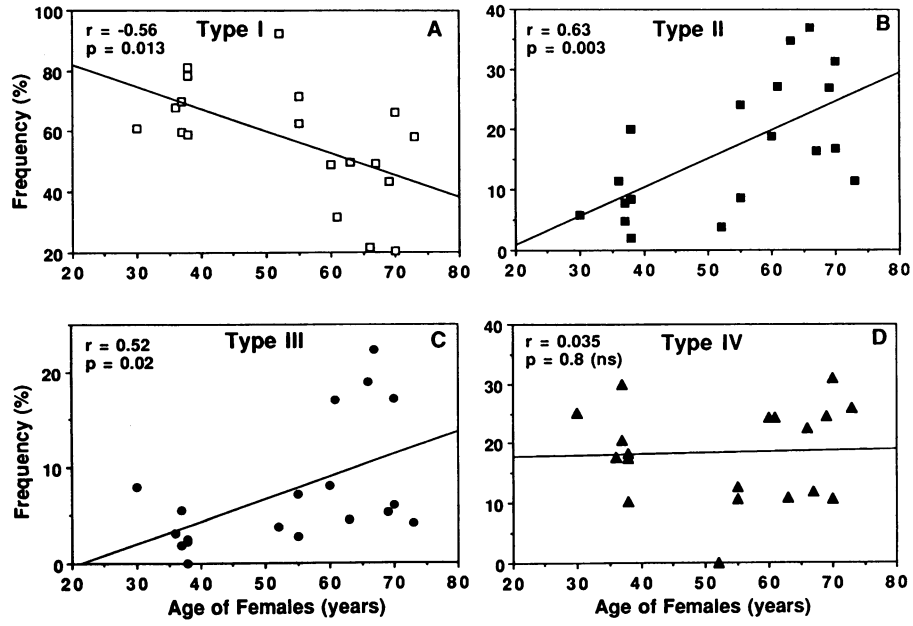

Figure 5. Relationship between the frequency of osteon types I, II, III, and IV with age in bone obtained from female subjects. Sections of bone were immunostained for osteocalcin and the proportion of osteon types was quantified (n = 19).

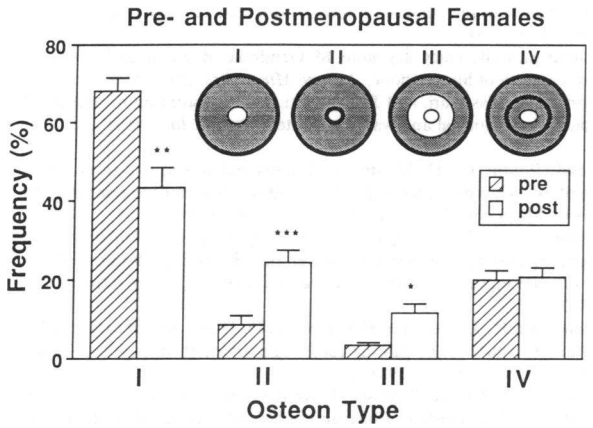

Figure 6. Frequency of osteon types in normal premenopausal (age, ≤ 40 yr; $n = 7$) and postmenopausal (age, ≥ 60 yr; $n = 9$) women. The frequency represents the percentage of the total number of osteons in each section of bone. Values represent the mean frequency ±SEM for each group. The actual number (mean±SEM) of osteons per bone section from pre- vs. postmenopausal females was 84.4±17.8 and 68.3±9.5, respectively. $*P = 0.01$; $**P = 0.003$; $***P = 0.001$ compared with premenopausal females.

The distribution of osteocalcin in type III osteons may be indicative of a delay in the incorporation of osteocalcin in these osteons. The presence of immunodetectable osteocalcin in the outer lamellae of osteons and its absence in the inner aspects may reflect the maturational state of the osteon. It is possible that type III osteons may, in time, become type I or II as osteocalcin accumulates in the extracellular matrix. Perhaps the morphologic pattern of osteocalcin in osteons of male and female bone is due to alterations in the endocrine milieu associated with age or gender that affect bone remodeling. Subsequently, these site-specific biochemical changes in osteocalcin may, in turn, impart altered bone remodeling.

Considering the limitations of immunohistochemical analyses, it cannot be concluded that differences in the apparent concentration of osteocalcin at specific sites in bone matrix are of cause or effect. However, previous studies suggest that the concentration of osteocalcin within bone matrix may be influenced by alterations in circulating hormones that occur with increased age. The expression of osteocalcin is stimulated by 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and further modulated by other local factors such as TGF-β (38–41). While some studies report decreased circulating levels of 1,25$(OH)_2D_3$ with age (42–44), similar findings were not observed by others (45–47). Nevertheless, an impaired ability of the aging kidneys to convert 25-hydroxyvitamin D_3 to 1,25-$(OH)_2$ D_3 may contribute to lower circulating levels with age (44, 46, 48). 1,25-$(OH)_2$ D_3 receptor levels in rats and in peripheral blood mononuclear cells of women are reduced with age (49, 50). Thus, a reduction in the incorporation of osteocalcin in bone matrix with age may be a reflection of reduced osteocalcin gene expression by 1,25-$(OH)_2D_3$ as a result of lower circulating levels of this sterol or reduced 1,25-$(OH)_2D_3$ receptor concentration in bone. Although circulating levels of parathyroid hormone increase with age in humans (51–53), this protein has not been shown to be a potent regulator of osteocalcin gene expression. Osteopetrosis in the rat is associated with reduced bone resorption, and matrix from these animals is deficient in osteocalcin (19). Combined with our findings, localized reductions in osteocalcin in the extracellular matrix of human bone may lead to site-specific reductions in cortical bone remodeling.

To determine the specificity of the immunohistochemical pattern of osteocalcin, we examined the patterns of other NCPs in adjacent histologic sections. In sections of bone stained for osteonectin, the pattern of distribution was similar to type III osteons with respect to the absence of stain in the matrix adjacent to Haversian canals (Fig. 1 *B* vs. Fig. 2 *C*, respectively). However, unlike type III osteons, which represented 3–20% of the total number of osteons, the pattern of osteonectin observed was consistent in all osteons and did not change with age or gender. The functional domain of osteocalcin contains two or three Gla residues that enhance binding of calcium ions and adsorption of this protein to hydroxyapatite (16, 54). These properties of osteocalcin suggest that this protein may regulate mineralization of bone matrix (14, 55–57). Osteonectin is a 38-kD glycoprotein with a high affinity for both type I collagen and hydroxyapatite, and is thought to promote mineralization by stabilizing hydroxyapatite and providing sites for nucleation of the mineral phase (58, 59). Considering both osteonectin and osteocalcin may regulate mineralization, it is interesting that the staining patterns are similar only in a small percentage of the osteons. Accordingly, differences in the distribution of osteocalcin may reflect site-specific and age-related differences in the mineralization of matrix. Further studies are needed to correlate microanatomical mineral density with the presence or absence of osteocalcin.

Histomorphometric analysis of adjacent sections of bone stained with Goldner's-Masson trichrome did not reveal any significant morphologic difference between type I, II, III, or IV osteons. In fact, there was no significant cellular activity within the Haversian canals. Morphologically, the osteons appear similar with respect to osteoblast and osteoclast number. These findings suggest that the observed patterns of distribution of immunodetectable osteocalcin are present in quiescent osteons.

Noncollagenous proteins in the extracellular matrix of bone are degraded with time and this process increases with advancing age (32, 60). The absence of osteocalcin in the matrix adjacent to Haversian canals as noted in type III osteons may reflect site-specific breakdown of protein. However, the absence of immunostaining in the inner lamellae of osteons was observed only when sections were stained for osteonectin and osteocalcin but not osteopontin or other NCPs in adjacent sections (13). This suggests that if degradative processes are responsible for the absence of osteocalcin staining in certain osteons, they would be highly protein and site specific.

In vivo, the activity of osteoblasts and the rate of bone deposition decreases with increasing age (61). It is unlikely that the changes in osteocalcin distribution are the result of a progressive decrease in the functional capacity of osteoblasts with advanced age as osteoblast-like cultures obtained from bone of aged donors respond to hormones and cytokines and are capable of producing mineralized matrix (62, 63). Furthermore, in this study the patterns of distribution of other NCPs such as osteopontin (Fig. 1 *A*), osteonectin (Fig. 1 *B*), and decorin (Fig. 1 *C*) were unchanged with age. These observations suggest that changes in cell modulators such as circulating hormones, local factors, and matrix signals are more likely responsible for the altered cellular activity observed with age.

The presence of resting (arrest) lines in bone reflects a period of cessation of osteoblast activity followed by commence-

ment of matrix deposition (64). The number of osteons with resting lines increases with age (65). In this study, there was a significant correlation between the proportion of osteons displaying resting lines (type IV) and age in males. The latter findings suggests that the deposition of bone in older males has a higher propensity to undergo periods of arrest. Alternatively, the incorporation of osteocalcin into arrest lines may increase with age in males. A similar age-related increase in type IV osteons was not observed in aging females. It is interesting to speculate that these gender-related differences observed in the distribution of osteocalcin may reflect estrogen depletion in the normal postmenopausal female. Interestingly, while the proportion of type IV osteons in females was higher than that of young males, by the seventh decade of life in males, the proportion of osteons with osteocalcin-positive resting lines exceeds that of females.

The complex organization of bone suggests that regulation of remodeling involves several levels of control. Although the maintenance of mineral homeostasis relies on circulating hormones such as 1,25-$(OH)_2D$ and parathyroid hormone, regulation of resorption sites must ultimately rely on microenvironmental signals such as cytokines generated by bone cells and on site-specific immobilized signals present within the extracellular matrix (8, 17, 60, 66). The localization of certain NCPs to cement lines, Haversian canals, and osteoclast attachment sites provides evidence that these proteins influence cell–matrix interactions (13, 20–22, 67, 68). In conclusion, the results of this study suggest that bone is heterogeneous with respect to the distribution of osteocalcin in osteons and that age- and gender-related changes in the distribution of osteocalcin in matrix may influence sites of remodeling and/or rates of mineralization.

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References

1. Thompson, D. 1980. Age changes in bone mineralization, cortical thickness, and haversian canal area. *Calcif. Tissue Int.* 31:5–11.

2. Riggs, B., H. Wahner, W. Dunn, R. Mazess, K. Offord, and L. Melton III. 1981. Differential changes in bone mineral density of the appendicular and axial skeleton with aging: relationship to spinal osteoporosis. *J. Clin. Invest.* 67:328–335.

3. Mazess, R. 1982. On aging bone loss. *Clin. Orthop. Relat. Res.* 165:239–252.

4. Huffer, W. 1988. Biology of disease: morphology and biochemistry of bone remodeling: possible control by vitamin D, parathyroid hormone, and other substances. *Lab Invest.* 59:418–442.

5. Noda, M., and G. Rodan. 1989. Transcriptional regulation of osteopontin production in rat osteoblast-like cells by parathyroid hormone. *J. Cell Biol.* 108:713–718.

6. Ignotz, R. 1991. TGF-β and extracellular matrix related influences on gene expression and phenotype. *Crit. Rev. Eukaryotic Gene Expression.* 1:75–84.

7. Sandberg, M. 1991. Matrix in cartilage and bone development: current views on the function and regulation of major organic components. *Ann. Med.* 23:207–217.

8. Finklemen, R., T. Linkhart, S. Mohan, K. Lau, D. Baylink, and N. Bell. 1991. Vitamin D deficiency causes a selective reduction in deposition of transforming growth factor β in rat bone: possible mechanism for impaired osteoinduction. *Proc. Natl. Acad. Sci. USA.* 88:3657–3660.

9. Groessner-Schreiber, B., M. Krukowski, C. Lyons, and P. Osdoby. 1992. Osteoclast recruitment in response to human bone matrix is age related. *Mech. Ageing Dev.* 62:143–154.

10. Ninomiya, J., R. Tracy, J. Calore, M. Gendreau, R. Kelm, and K. Mann. 1990. Heterogeneity of human bone. *J. Bone Miner. Res.* 5:933–938.

11. Lian, J., M. Tassinari, and J. Glowacki. 1984. Resorption of implanted bone prepared from normal and warfarin-treated rats. *J. Clin. Invest.* 73:1223–1226.

12. VanderPluijm, G., H. Mouthaan, S. Papapoulos, and C. Lowik. 1991. Inhibition of osteoclast precursor attachment and subsequent bone resorption by synthetic RGD-peptides. *J. Bone Miner. Res.* 6:S146 (Abstr.).

13. Ingram, R., B. Clarke, L. Fisher, and L. Fitzpatrick. 1993. Distribution of noncollagenous matrix proteins in the extracellular matrix of adult human bone: evidence of anatomical and functional heterogeneity. *J. Bone Miner. Res.* 8:1019–1029.

14. Hauschka, P., J. Lian, and P. Gallop. 1975. Direct identification of the calcium binding amino acid, gamma-carboxyglutamate in mineralized tissue. *Proc. Natl. Acad. Sci. USA.* 72:3925–3929.

15. Price, P., A. Otsuka, J. Poser, J. Kristanponis, and N. Raman. 1976. Characterization of a gamma-carboxyglutamic acid-containing protein from bone. *Proc. Natl. Acad. Sci. USA.* 73:1447–1451.

16. Hauschka, P. 1982. Osteocalcin: the vitamin K-dependent calcium-binding protein of bone matrix. *In* Heritable Disorders of Connective Tissue. W. Akeson, P. Bornstein, and M. Glimcher, editors. CV Mosby, St. Louis. 195–207.

17. Glowacki, J., and J. Lian. 1987. Impaired recruitment and differentiation of osteoclast progenitors by osteocalcin-deplete bone implants. *Cell Differ.* 21:247–254.

18. DeFranco, D., J. Glowacki, K. Cox, and J. Lian. 1991. Normal bone particles are preferentially resorbed in the presence of osteocalcin-deficient bone particles *in vivo*. *Calcif. Tissue Int.* 49:43–50.

19. Lian, J., and A. Marks, Jr. 1990. Osteopetrosis in the rat: Coexistence of reductions in osteocalcin and bone resorption. *Endocrinology.* 126:955–962.

20. Reinholt, F., K. Hultenby, A. Oldberg, and D. Heinegard. 1990. Osteopontin: a possible anchor of osteoclasts to bone. *Proc. Natl. Acad. Sci. USA.* 87:4473–4475.

21. Carlson, C., H. Tulli, M. Jayo, R. Loeser, R. Tracy, K. Mann, and M. Adams. 1993. Immunolocalization of noncollagenous bone matrix proteins in lumbar vertebrae from intact and surgically menopausal cynomolgus monkeys. *J. Bone Miner. Res.* 8:71–81.

22. McKee, M., M. Glimcher, and A. Nanci. 1992. High-resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. *Anat. Rec.* 234:479–492.

23. Bolander, M., P. Robey, L. Fisher, K. Conn, B. Prabhakar, and J. Termine. 1989. Monoclonal antibodies against osteonectin show conservation of epitopes across species. *Calcif. Tissue Int.* 45:74–80.

24. Fisher, L., J. Termine, and M. Young. 1989. Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. *J. Biol. Chem.* 264:4571–4576.

25. Fisher, L., G. Hawkins, N. Tuross, and J. Termine. 1987. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. *J. Biol. Chem.* 262:9702–9708.

26. Bianco, P., L. Fisher, M. Young, J. Termine, and P. Robey. 1990. Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues. *J. Histochem. Cytochem.* 38:1549–1563.

27. Weinreb, M., D. Shinar, and G. Rodan. 1990. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. *J. Bone Miner. Res.* 5:831–842.

28. Stein, G., J. Lian, and T. Owen. 1990. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:3111–3123.

29. Kahn, A., S. Teitelbaum, J. Malone, and M. Krukowski. 1982. The relationship of monocyte cells to the differentiation and resorption of bone. *Prog. Clin. Biol. Res.* 110B:239–248.

30. Malone, J., S. Teitelbaum, G. Griffin, R. Senior, and A. Kahn. 1982. Recruitment of osteoclast precursors by purified bone matrix constituents. *J. Cell Biol.* 92:227–235.

31. Dickson, I., and M. Bagga. 1985. Changes with age in the non-collagenous proteins of human bone. *Connect. Tissue Res.* 14:77–85.

32. Conn, K., and J. Termine. 1985. Matrix protein profiles in calf bone development. *Bone (Elmsford).* 6:33–36.

33. Oldberg, A., A. Franzen, and D. Heinegard. 1986. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc. Natl. Acad. Sci. USA.* 83:8819–8823.

34. Hynes, R. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

35. Horton, M., and J. Davies. 1989. Perspectives: adhesion receptors in bone. *J. Bone Miner. Res.* 4:803–808.

36. Helfrich, M., S. Nesbitt, E. Dorey, and M. Horton. 1992. Rat osteoclasts

adhere to a wide range of RGD (Arg-Gly-Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via $a_v \beta_3$ Integrin. *J. Bone Miner. Res.* 7:335–343.

37. Hruska, K., F. Rolnick, and M. Huskey. 1992. Occupancy of the osteoclast $\partial_v \beta_3$ integrin by osteopontin stimulates a novel SRC associated phosphatidylinositol 3 kinase (P13 kinase) resulting in phosphatidylinositol trisphosphate (PIP_3) formation. *J. Bone Miner. Res.* 7:S106.

38. Lian, J., C. Stewart, E. Puchacz, S. Mackowiak, V. Shalhoub, D. Collart, G. Zambetti, and G. Stein. 1989. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. *Proc. Natl. Acad. Sci. USA.* 86:1143–1147.

39. Noda, M. 1989. Transcriptional regulation of osteocalcin production by transforming growth factor-β in rat osteoblast-like cells. *Endocrinology.* 124:612–617.

40. Osono, K., J. Liao, S. Kerner, R. Scott, and J. Pike. 1990. The vitamin D-response element in the human osteocalcin gene. *J. Biol. Chem.* 265:21881–21888.

41. Staal, A., H. Pols, C. Buurman, T. Vink, G. VandenBemd, J. Birkenhager, and J. vanLeeuwen. 1992. Differences in interaction between TGFβ and $1,25(OH)_2D_3$ in VDR and osteocalcin gene expression. *J. Bone Miner. Res.* 7:S173.

42. Chapuy, M. C., F. Durr, and P. Chapuy. 1983. Age-related changes in parathyroid hormone and 25 hydroxycholecalciferol levels. *J. Gerontol.* 38:19–22.

43. Omdahl, J., P. Garry, L. Hunsaker, W. Hunt, and J. Goodwin. 1982. Nutritional status in a healthy elderly population: vitamin D. *Am. J. Clin. Nutr.* 36:1225–1233.

44. Tsai, K. S., H. Heath III, R. Kumar, and B. Riggs. 1984. Impaired vitamin D metabolism with aging in women: possible role in pathogenesis of senile osteoporosis. *J. Clin. Invest.* 73:1668–1672.

45. Clemens, T., X. Y. Zhou, M. Myles, D. Endres, and R. Lindsay. 1986. Serum vitamin D_2 and vitamin D_3 metabolite concentrations and absorption of vitamin D_2 in elderly subjects. *J. Clin. Endocrinol. & Metab.* 63:656–660.

46. Halloran, B., A. Portale, E. Lonergan, and R. Curtis-Morris, Jr. 1990. Production and metabolic clearance of 1,25 dihydroxyvitamin D in men: effect of advancing age. *J. Clin. Endocrinol. & Metab.* 70:318–323.

47. Hartwell, D., B. Riis, and C. Christiansen. 1990. Changes in vitamin D metabolism during natural and medical menopause. *J. Clin. Endocrinol. & Metab.* 71:127–132.

48. Buchanan, J., C. Myers, and R. Greer. 1988. Effect of declining renal function on bone density in aging women. *Calcif. Tissue Int.* 43:1–6.

49. Horst, R., J. Goff, and T. Reinhardt. 1990. Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. *Endocrinology.* 126:1053–1057.

50. Olmos, J., J. Amado, J. Riancho, M. Albajar, and J. Macias-Gonzalez. 1990. Sex and age distribution of 1,25(OH)2D3 receptors in peripheral blood mononuclear cells from normal human subjects. *Bone (Elmsford).* 11:407–409.

51. Orwoll, E., and D. Meier. 1986. Alterations in calcium, vitamin D, and parathyroid hormone physiology in normal men with aging: Relationship to the development of senile osteopenia. *J. Clin. Endocrinol. & Metab.* 63:1262–1269.

52. Quesada, J., W. Coopmans, B. Ruiz, P. Aljama, I. Jans, and R. Bouillon. 1992. Influence of vitamin D on parathyroid function in the elderly. *J. Clin. Endocrinol. & Metab.* 75:494–501.

53. Chan, E., E. Lau, C. Shek, D. MacDonald, J. Woo, P. Leung, and R. Swaminathan. 1992. Age-related changes in bone density, serum parathyroid hormone, calcium absorption and other indices of bone metabolism in Chinese women. *Clin. Endocrinol.* 4:375–381.

54. Poser, J., and P. Price. 1979. A method for decarboxylation of gamma-carboxyglutamic acid in proteins: Properties of the decarboxylated gamma-carboxyglutamic acid from calf bone. *J. Biol. Chem.* 254:431–436.

55. Termine, J., A. Belcourt, K. Conn, and H. Kleinman. 1981. Mineral and collagen binding proteins of fetal calf bone. *J. Biol. Chem.* 256:10403–10408.

56. Termine, J. 1986. Bone proteins and mineralization. *Rheumatology.* 10:184–196.

57. Hauschka, P., and F. Wians, Jr. 1989. Osteocalcin-hydroxyapatite interaction in the extracellular organic matrix of bone. *Anat. Rec.* 224:180–188.

58. Termine, J., H. Kleinman, S. Whitson, K. Conn, M. McGarvey, and G. Martin. 1981. Osteonectin, a bone-specific protein linking mineral to collagen. *Cell.* 26:99–105.

59. Fisher, L., and J. Termine. 1985. Noncollagenous proteins influencing the local mechanisms of calcification. *Clin Orthop. Relat. Res.* 200:362–385.

60. Termine, J. 1990. Cellular activity, matrix proteins, and aging bone. *Exp. Gerontol.* 25:217–221.

61. Meunier, P. 1988. Assessment of bone turnover by histomorphometry in osteoporosis. *In* Osteoporosis, Etiology, Diagnosis and Management. B. Riggs and L. Melton III, editors. Raven Press, Ltd., New York. 317–332.

62. Robey, P. G., and J. Termine. 1985. Human bone cells *in vitro. Calcif. Tissue Int.* 37:453–460.

63. Koshihara, Y., M. Hirano, M. Kawamura, H. Oda, and S. Higaki. 1991. Mineralization ability of cultured human osteoblast-like periosteal cells does not decline with aging. *J. Gerontol.* 46:201–206.

64. Parfitt, A. 1983. The physiological and clinical significance of bone histomorphometric data. *In* Bone Histomorphometry: Techniques and Interpretation. R. R. Recker, editor. CRC Press, Inc., Boca Raton. 143–223.

65. Pankovich, A., D. Simmons, and V. Kulkarni. 1974. Zonal osteons in cortical bone. *Clin. Orthop. Relat. Res.* 100:356–363.

66. Yamaguchi, Y., D. Mann, and E. Ruoslahti. 1990. Negative regulation of transforming growth factor-β by the proteoglycan decorin. *Nature (Lond.).* 346:281–284.

67. Hultenby, K., F. Reinholt, A. Oldberg, and D. Heingard. 1991. Ultrastructural immunolocalization of osteopontin in metaphyseal and cortical bone. *Matrix.* 11:206–213.

68. McKee, M., M. Farach-Carson, W. Butler, P. Hauschka, and A. Nanci. 1993. Ultrastructural immunolocalization of noncollagenous (osteopontin and osteocalcin) and plasma (albumin and alpha2HS-glycoprotein) proteins in rat bone. *J. Bone Miner. Res.* 8:485–496.