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Age-Related Changes in Bone Formation, Osteoblastic Cell Proliferation, and Differentiation During Postnatal Osteogenesis in Human Calvaria

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Abstract We have determined the age-related changes in the growth characteristics and expression of the osteoblast phenotype in human calvaria osteoblastic cells in relation with histologic indices of bone formation during postnatal calvaria osteogenesis. Histomorphometric analysis of normal calvaria samples obtained from 36 children, aged 3 to 18 months, showed an age-related decrease in the extent of bone surface covered with osteoblasts and newly synthesized collagen, demonstrating a progressive decline in bone formation during postnatal calvaria osteogenesis. Immunohistochemical analysis showed expression of type I collagen, bone sialoprotein, and osteonectin in the matrix and osteoblasts, with no apparent age-related change during postnatal calvaria osteogenesis. Cells isolated from human calvaria displayed characteristics of the osteoblast phenotype including alkaline phosphatase (ALP) activity, osteocalcin (OC) production, expression of bone matrix proteins, and responsiveness to calciotropic hormones. The growth of human calvaria osteoblastic cells was high at 3 months of age and decreased with age, as assessed by (^{3}H)-thymidine incorporation into DNA. Thus, the age-related decrease in bone formation is associated with a decline in osteoblastic cell proliferation during human calvaria osteogenesis. In contrast, ALP activity and OC production increased with age in basal conditions and in response to 1,25$(OH)_2$ vitamin D_3, suggesting a reciprocal relationship between cell growth and expression of phenotypic markers during human postnatal osteogenesis. Finally, we found that human calvaria osteoblastic cells isolated from young individuals with high bone formation activity in vivo and high growth potential in vitro had the ability to form calcified nodular bone-like structures in vitro in the presence of ascorbic acid and β-glycerophosphate, providing a new model to study human osteogenesis in vitro. J. Cell. Biochem. 64:128–139.

Key words: osteoblasts; calvaria; bone formation; proliferation; differentiation; osteogenesis

In higher vertebrates, part of the skull derives from the cephalic mesoderm, whereas frontal and parietal bones originate from the neural crest [Le Lièvre, 1978; Couly et al., 1993]. Skull development in the late fetal life is characterized by mesenchyme ossification which involves the continuous apposition of bone matrix by differentiated osteoblasts and formation of a network of connecting trabeculae [Markens, 1975; Persson et al., 1978]. The normal postnatal development of the skull requires continuous osteogenesis at facial and cranial sutures until their fusion [Persson et al., 1978; Cohen, 1993]. The cellular and molecular mechanisms that are involved in the development of osteogenesis in human calvaria have not been identified.

Osteogenic differentiation in rodents has been extensively studied in vitro using osteoblast precursor cells derived from the calvaria [Ecarot-Charrier et al., 1983; Nefussi et al., 1985; Bellows et al., 1986], endosteal bone [Lomri et al., 1988; Modrowki and Marie, 1993], or marrow stroma [Maniatopoulos et al., 1988; Benayahu et al., 1989; Malaval et al., 1994]. When cultured in conditions that facilitate the formation of a mineralized bone matrix, rat calvaria cells form mineralized bone-like nodules [Nefussi et al., 1985; Bellows et al., 1986], and this model has been used to determine the temporal expression of bone-related proteins during osteogenesis [Owen et al., 1990]. Osteogenesis induced by rat calvaria cells in vitro is a

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time- and spatially-regulated process characterized by initial proliferation of osteoprogenitor cells that declines with time, the progressive differentiation of osteoblasts associated with the expression of alkaline phosphatase, an early marker of the osteoblast phenotype, and the deposition of a collagenous extracellular matrix which is followed by increasing expression of osteocalcin, a late marker of differentiated osteoblasts [Owen et al., 1990]. In contrast to rodent bone cells, human osteoblastic cells isolated from the endosteum or the marrow stroma do not readily form bone-like nodules in vitro even if the cells express the messages and proteins for bone matrix [Beresford et al., 1993; Cheng et al., 1994]. The sequence of cellular and molecular events involved in osteogenesis induced by human osteoblasts in vitro remains therefore to be determined.

In the present study, we have analyzed the growth characteristics and phenotype of human calvaria osteoblastic cells in vitro in comparison with the age-related changes in bone formation during postnatal calvaria osteogenesis in humans. We report here that the age-related decrease in bone formation during postnatal calvaria osteogenesis is associated with a decrease in osteoblastic cell proliferation followed by increased expression of phenotypic parameters, and that human calvarial cells isolated from young individuals with high bone formation activity in vivo and high osteoblastic cell growth potential have the ability to form bone-like structures in vitro.

MATERIALS AND METHODS

Bone Samples

Normal human calvaria bone samples were obtained from 36 infants, aged 3 to 18 months, during local surgery after informed consent and approval by our Human Experimentation Committee. Normal bone samples were obtained at the time of surgery performed for cranial reconstruction or tumor resection. The samples consisted of small amounts of normal calvaria obtained close to, and including the saggital, metopic, or coronal sutures. The samples were sectioned into two to three parts under sterile conditions. A bone fragment was fixed in 70% ethanol and embedded undecalcified in glycol methacrylate, as described [Hott and Marie, 1987]. Other fragments were used for cell cultures, as described below. When available, fragments of bone samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, decalcified in buffered EDTA (pH 7.2) before embedding in paraffin for immunohistochemistry of bone matrix proteins [Bianco et al., 1991].

Histomorphometrical and Histochemical Analysis

Longitudinal sections (5 μm thick) of plastic-embedded bone samples were stained with Goldner trichrome or Von Kossa to identify bone forming sites and bone cells [Baron et al., 1983], or were stained histochemically for alkaline phosphatase activity [Hott and Marie, 1987]. For each bone sample, histomorphometric indices of bone formation were measured using an ocular integrator coupled to a microscope (BH-2 Olympus, Tokyo, Japan) using conventional methods [Baron et al., 1983; Parfitt et al., 1987]. The following indices were measured: the osteoid surface (% bone surface covered with uncalcified bone matrix) and the osteoblast surface (% bone surface covered with osteoblasts).

Immunohistochemical Analysis

The age-related changes in the expression of bone matrix proteins were determined in human calvaria samples obtained from 12 subjects aged 4–30 months. Longitudinal sections (5 μm thick) of decalcified paraffin-embedded bone samples were washed in PBS, treated with 3% BSA for 15 min to saturate the nonspecific binding sites, washed in PBS and exposed to 3% H_2O_2 for 30 min to eliminate the endogenous peroxydase activity. After washing in PBS, the tissues were exposed to one of the following specific antisera (generously provided by Dr. L.W. Fisher, NIDR, NIH, USA) [Bianco et al., 1991; Fisher, 1995] used at 1/100 dilution: antibovine bone sialoprotein (BSP) crossing to human BSP (LF-6), antihuman C-telopeptide (synthetic) of α1(I) collagen (LF-67), and antihuman osteonectin (synthetic peptide) (LF-37) raised in the rabbit. Control sections were treated with non-immune serum used at the same dilution instead of the primary antibody. After exposure for 1 h at room temperature the antiserum was removed, the sections were washed three times for 10 min in PBS and exposed to a second anti-rabbit donkey antibody (1/100) linked to horseradish peroxydase (Boehringer, Mannheim, Germany) for 1 h. After washing in PBS, the specific antigen-antibody binding was revealed by exposing the cells to 1 mg/ml diaminobenzidin in 10 mM Tris buffer (pH 7.6) and 0.05% H_2O_2 for 20 min at

room temperature. The immunohistochemical staining was visualized by light microscopy.

Osteoblastic Cell Cultures

Osteoblastic cells were obtained from the normal human calvaria samples by collagenase digestion. The bone samples were washed in PBS, dissected in about 1 mm^3 size fragments which were treated with 0.25% collagenase (Type I, Sigma, St Louis, MO) for 2 h at 37°C. The collagenase-treated bone fragments were washed in Dulbecco's modified essential medium (DMEM) supplemented with glutamine (292 mg/L), 10% heat inactivated fetal calf serum (FCS), 1% antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). After several washes, the cells were collected by centrifugation, suspended in DMEM, plated in 25 cm^2 flasks, and cultured in DMEM supplemented with 10% FCS at 37°C. At confluence, the cells were detached with 0.1% trypsin/EDTA, counted, and plated at the density indicated below. All cell populations were cultured under identical conditions. Because long term cultures and successive passages induce reduction of the osteoblast phenotype [Marie et al., 1989], only first-passaged cells were used. The cells obtained using this method were found to express characteristics of the osteoblast phenotype [de Pollak et al., 1996].

Osteoblastic Cell Characteristics

To further determine the characteristics of cells derived from the normal human calvaria samples, the expression of bone matrix proteins was evaluated by immunocytochemistry. The cells were plated at 5,000 cells/cm^2 on multiwell chambers (LabTek, Nunc, Naperville) and cultured until pre-confluency (5 days). The cells were then washed in PBS, treated with 3% BSA for 15 minutes to saturate the nonspecific binding sites, washed, and exposed to 3% H_2O_2 for 30 min to eliminate endogenous peroxydase activity. After washing in PBS, the cells were exposed to one of the following specific antisera (generously provided by Dr. L.W. Fisher, NIDR, NIH, USA) [Bianco et al., 1990, 1991; Fisher, 1995] used at 1/200 dilution: antibovine osteocalcin (LF-32), antiosteonectin (LF-37), antihuman biglycan (LF-15), and antihuman α1(I) collagen (LF-67). Control cells were treated with non-immune serum used at the same dilution instead of the primary antibody. Immunostaining was then revealed as described above. The immunocytochemical staining was visualized by light microscopy.

To determine the osteogenic capacity of osteoblastic cells derived from human calvaria samples, cells isolated from individuals at different ages (3–6 months) were plated at high density (2.10^5–1.10^6 cells/cm^2) and cultured in the presence of 50 µg/ml ascorbic acid and 3 mM β glycerophosphate, conditions that induce osteogenesis in calvarial [Nefussi et al., 1985; Bellows et al., 1986; Owen et al., 1990] and endosteal cell cultures [Lomri et al., 1988; Modrowski and Marie, 1993]. After 21 days of culture, half of the cultures were harvested, fixed in 70% ethanol, and embedded undecalcified in glycolmethacrylate [Hott and Marie, 1987]. Thin (5 µm) histologic sections were obtained and stained with Von Kossa for detection of minerals, or with toluidine blue to visualize the matrix. The other half was used to determine the expression of type I collagen. These cultures were washed in PBS, fixed in 4% PFA in PBS at 4°C and reacted immunocytochemically for type I collagen, as described above.

DNA Synthesis

The proliferative capacity of osteoblastic cells isolated from the human calvaria samples was evaluated by [^{3}H]-thymidine incorporation into DNA, as described [Marie et al., 1989]. Cells plated at 10,000 cells/cm^2 in 24-well plates were cultured in DMEM with 10% FCS for 14 days. The cells were labeled with 2 µCi/well of 6-[^{3}H]-thymidine on days 0, 2, 6, 8, and 13 and [^{3}H]-thymidine incorporation into DNA was determined 24 h later on days 1, 3, 7, 9, and 14. At these time-points, the cell layer was collected by trypsinization, DNA was precipitated with thrichloracetic acid (TCA), the TCA-insoluble fraction was dissolved in NaOH, and [^{3}H]-thymidine incorporation into DNA was measured in three aliquots by liquid scintillation. The time-course of cell growth was evaluated in each culture obtained from individual subjects and parameters of cell growth (peak of incorporation and cumulative incorporation of [^{3}H]-thymidine into DNA) were obtained from the profile of DNA synthesis during the time-course study, as described previously [Marie et al., 1989, 1991].

Biochemical Determinations

Alkaline phosphatase (ALP) activity and osteocalcin production were determined in each

osteoblastic cell culture, as described [Marie et al., 1989, 1991]. Cells plated at 10,000 cells/cm^2 in 6-well plates were cultured in DMEM with 10% FCS until confluence. The medium was removed and the cells were cultured in the presence of 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D$) at the indicated concentration, or its solvent. After 48 h, the medium was removed and frozen for osteocalcin determination. The cells were rinsed in cold PBS, scraped in distilled water, and sonicated. ALP activity in the cell lysates were determined by a colorimetric method using phenyl phosphate as substrate (Biomerieux, Craponne, France). The protein content of the cell lysates was determined colorimetrically (Bio-Rad, Ivry, France). The activity of the enzyme was expressed as nanomoles of *p*-nitrophenol released per min/mg protein. The osteocalcin concentration in the medium was measured in duplicate by RIA (Cis-Oris, Gif sur Yvette, France) using a specific antibody raised against bovine osteocalcin. The maximum inter- and intra-assay coefficients of variation for the range of concentrations evaluated were 6.1% and 3.4%, respectively, and the lower detection limit of the assay was 0.35 µg/liter. The production of osteocalcin in the medium was evaluated in each culture and was corrected for cell protein.

Statistics

The data are expressed as mean ± SEM. Differences between the mean values in the different groups were analyzed using the statistical package super-ANOVA (Macintosh, Abacus concepts, Inc., Berkeley, CA) with a minimal significance of $P < 0.05$.

RESULTS

Age-Related Changes in Postnatal Calvarial Bone Formation

At the early step of postnatal calvarial osteogenesis, human calvaria consisted mainly of trabecular bone surrounded by cortical plates. The histological examination revealed that bone formation was very active along the bone surface in both trabecular bone (Fig. 1a) and cortical bone (Fig. 1b). Numerous osteoblasts (Fig. 1c) which were alkaline phosphatase-positive (Fig. 1d) were disposed along the bone surface, showing the high bone forming activity along the bone matrix. The histological examination revealed that the number of bone forming cells decreased rapidly with age after birth, and the age-related change in bone formation was then determined by histomorphometric analysis. The quantitative analysis of static indices of bone formation (extent of bone surface covered with osteoid and osteoblasts) showed a decrease in bone formation parameters from 3 to 18 months of age (Fig. 2a,b).

The immunohistochemical analysis showed a specific labeling for type I collagen (Fig. 3b), bone sialoprotein (Fig. 3c), and osteonectin (Fig. 3d) in the human calvaria samples. Collagen type I was found abundant in the matrix (grey staining in Fig. 3b) and was strongly expressed in osteoblasts along the bone surface (black staining and arrows in Fig. 3b). Bone sialoprotein was found in the matrix (grey staining in Fig. 3c) and was also expressed in osteoblasts lining the bone matrix (arrows in Fig. 3c), although the staining was less intense than collagen type I in most samples (Table I). Osteonectin was found in the matrix (grey staining in Fig. 3d) and in osteoblasts (arrows in Fig. 3d). Some young osteocytes stained for these matrix proteins whereas unmineralized matrix was unlabeled (see for example Fig. 3c, small arrows). Figure 3a shows that the control sections exposed to a non-immune serum instead of the specific primary antibody had no staining in the matrix or in osteoblasts (arrows). In all calvaria samples obtained from 4 to 30 months of age, type I collagen was found to be intensely expressed in both bone matrix and osteoblasts (Table I). In contrast, labeling for osteonectin and bone sialoprotein was less intensely expressed than type I collagen. These changes in expression reflected some variations between individuals rather than differences with age (Table I).

Characteristics of Human Calvaria Osteoblastic Cells

Cells derived from normal postnatal calvaria were characterized in terms of expression of the osteoblast phenotype. In unstimulated conditions, about half of the cells at confluency showed intense alkaline phosphatase activity as revealed by cytochemistry (Fig. 4a) and a variable fraction of cells stained positively for bone matrix proteins. Collagen type I was expressed in about 30% of the cells (Fig. 4c), whereas osteocalcin (Fig. 4d), osteonectin (Fig. 4e), and biglycan (Fig. 4f) were found in about 10–20% of the cells in basal culture conditions,

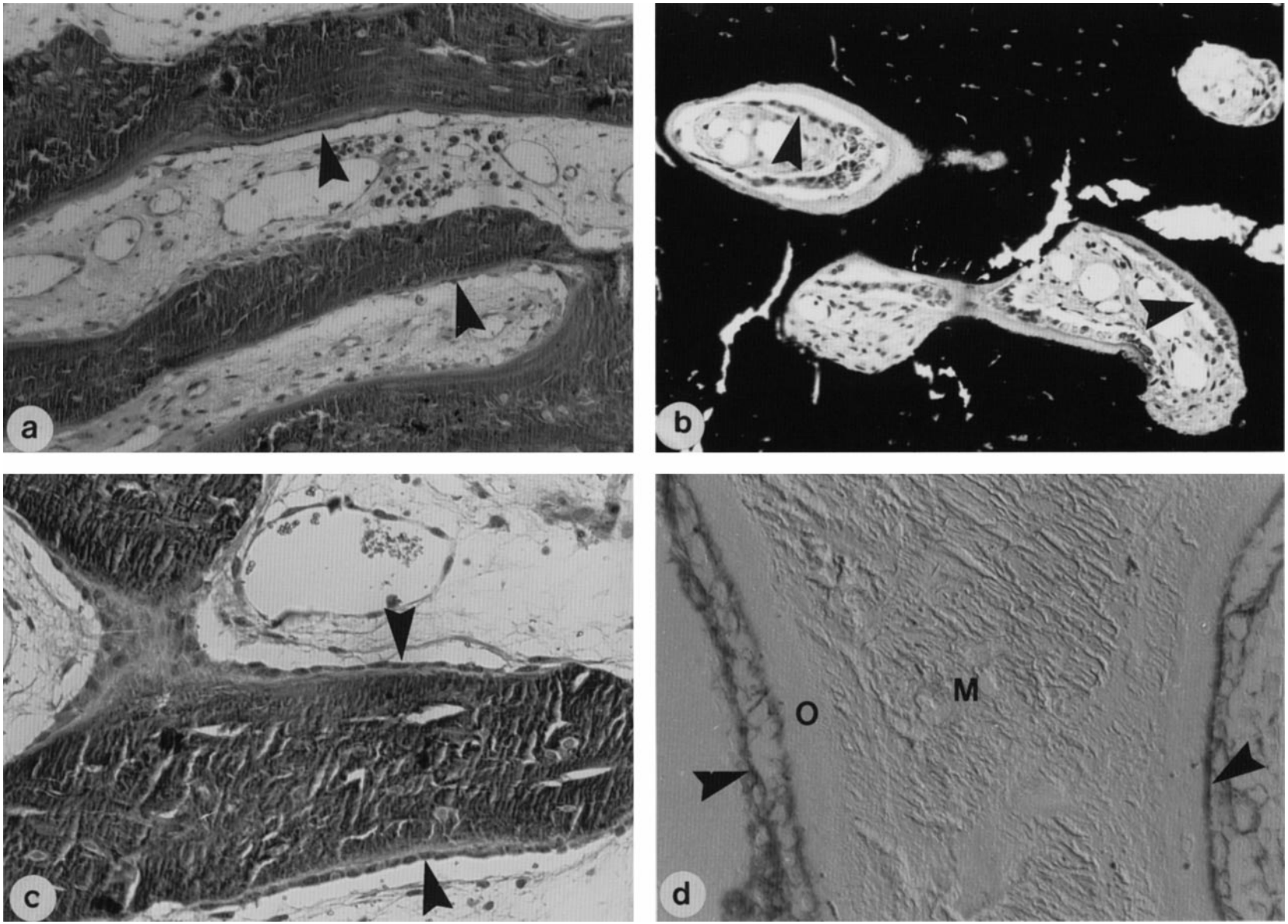

Fig. 1. Histological appearance of a normal 3-month-old human calvaria showing the high activity of bone formation. Five µm-thick sections were stained with Goldner trichrome (**a, c**), or von Kossa (**b**), or were unstained and cytochemically reacted for alkaline phosphatase activity (**d**). The bone sample shows numerous active bone forming sites in trabecular bone (a) and cortical plate (b) with active osteoblasts (arrows). Alkaline phosphatase-positive osteoblasts (d) are actively synthesizing osteoid (O) which becomes a mineralized bone matrix (M). (Magnification: ×250).

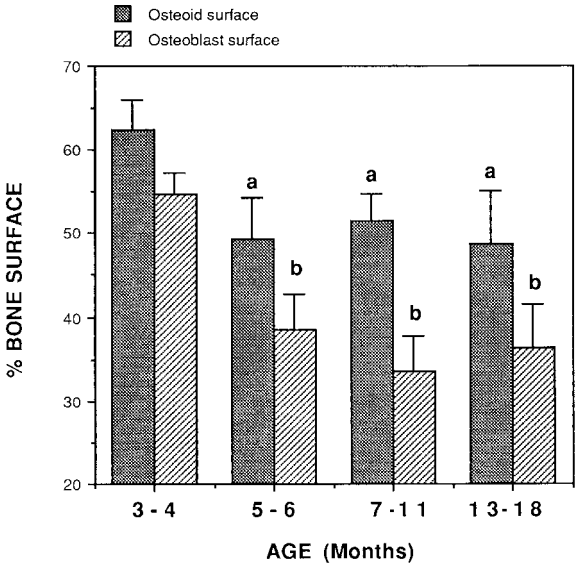

Fig. 2. Age-related decrease in histomorphometric indices of bone formation in human postnatal calvaria (n = 27). The data are the mean ± SEM of 3–10 samples per time point. **a, b:** A significant difference with the respective values at 3 months of age ($P < 0.05$).

as revealed by immunocytochemistry. Human calvaria osteoblastic cells were found to produce osteocalcin in the medium, and ALP activity and osteocalcin production were stimulated dose-dependently by $1,25(OH)_2D$ (not shown). The cells also responded dose-dependently to parathormone (PTH) with a 3.8-fold increase in cAMP at 10^{-7} M PTH, indicating that the cultures contained osteoblast precursor cells.

We found that some human calvaria cell cultures had the ability to form nodular structures in vitro, and this ability was related to the age of the subjects. Only cells obtained from young subjects who had high bone formation activity and high cell growth in vitro (see below) had the ability to form nodular structures in vitro. When cultured in the presence of ascorbic acid and β glycerophosphate, human calvaria cells obtained from young (≤3 months old) individuals and grown at confluence produced an abundant extracellular matrix composed of collagen type I as revealed by immunocytochemistry (not shown). In long term cultures, the extracellular matrix became organized in three-dimension, forming nodular structures (about 5/cm^2 flask at 21 days of culture) of various size. Histological analysis showed that the nodular structures presented some similarities with the typical nodules formed by rat or mouse calvaria cells (Fig. 5). The structures formed by human calvaria cells were composed of a woven bone-like matrix (Fig. 5a,c). Numerous ALP-positive cells were found around the matrix (Fig. 5d) and osteocyte-like cells were found embedded into this matrix (Fig. 5a,c). In addition, calcification of the nodular structures occurred progressively with time (Fig. 5b). These results indicate that human osteoblastic cells derived from young normal postnatal human calvaria have the ability to differentiate into osteogenic osteoblasts in vitro.

Age-Related Changes in Osteoblastic Cell Proliferation

Because the osteoblast phenotype varies with the age of the subject, we have determined the age-related changes in the proliferation of human calvaria osteoblastic cells in vitro. The analysis of the peak of DNA synthesis and the cumulative (^{3}H)-thymidine incorporation into DNA, two indices of cell proliferation [Marie et al., 1989, 1991], from 1 to 14 days of culture showed that cells obtained from younger individuals had the higher growth capacity. In addition, we found that the growth of human calvaria osteoblastic cell growth decreased with age from 3–4 months to 13–18 months of age (Fig. 6). Thus, human calvaria cell proliferation is initially high and decreased during osteogenesis.

Age-Related Changes in ALP Activity and Osteocalcin Production

We then determined the age-related changes in osteoblast markers in human calvaria osteoblastic cells. The biochemical analysis of alkaline phosphatase activity, an early marker of osteoblast differentiation, showed that the mean ALP activity increased with age from 3–4 months to 13–18 months of age (Fig. 7). The cells did not respond to 10 nM $1,25(OH)_2D$ at early postnatal time-points but responded significantly to 10 nM $1,25(OH)_2D$ at 7–11 months of age and thereafter (Fig. 7). The mean osteocalcin production by human calvaria osteoblastic cells was also found to increase from 3–6 months to 7–18 months of age (Fig. 8). In contrast to ALP activity, OC production was stimulated by $1,25(OH)_2D$ at all ages postnatally. These results indicate that alkaline phosphatase activity and osteocalcin production, early and late markers of osteoblastic cell differentiation, are initially low and increase during osteogenesis.

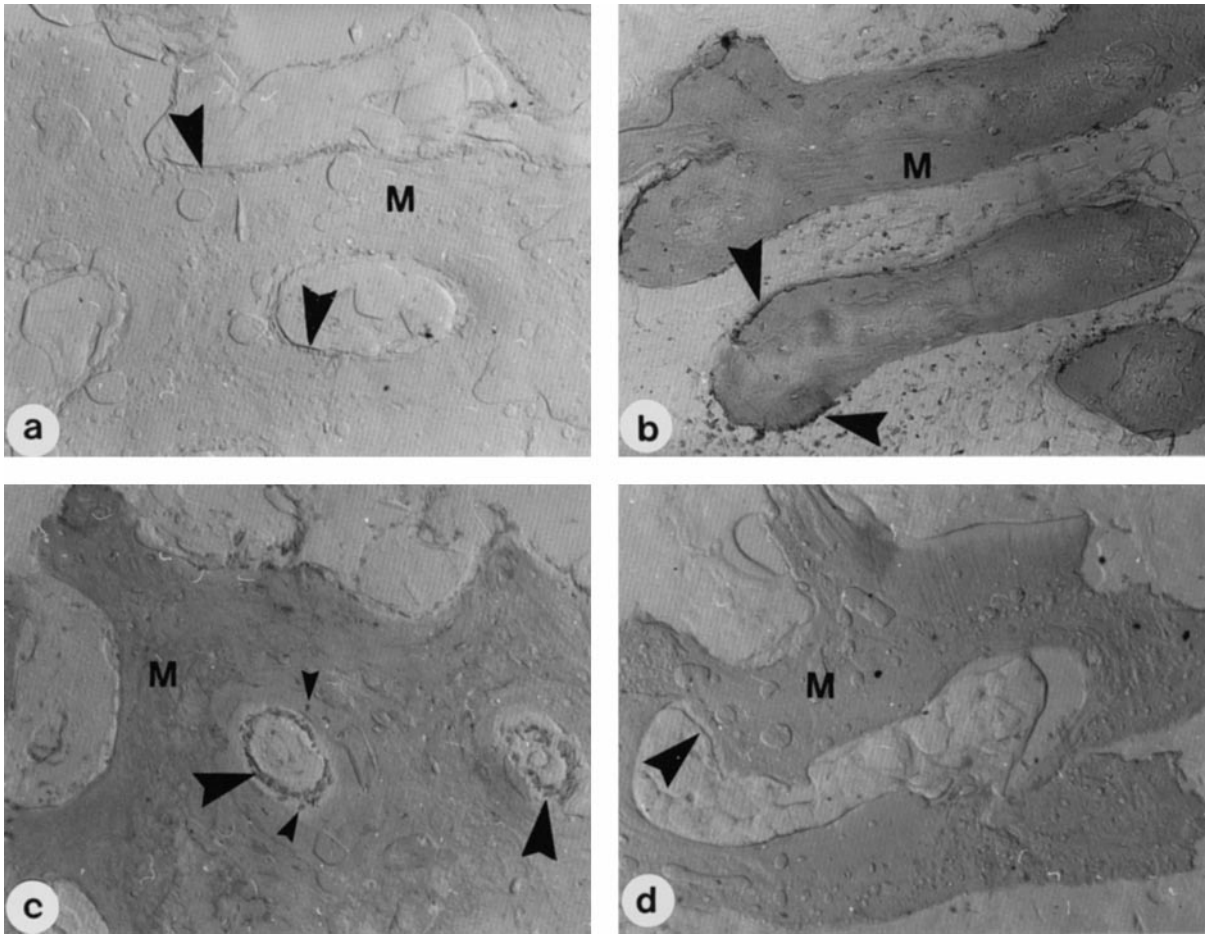

Fig. 3. Microphotographs of a representative section of a normal 3-month-old human calvaria showing the expression of bone matrix proteins by immunohistochemistry. The human calvaria shows a marked labeling for type I collagen (**b**) and less intense staining for bone sialoprotein (**c**) and osteonectin (**d**). The bone matrix (M), osteoblasts (arrows) and some osteocytes (small arrows) were found to be labeled. Control section incubated with non-immune serum (**a**) shows no labeling in the matrix or osteoblasts (arrows) (magnification: ×125).

DISCUSSION

The results of this study indicate that the age-related decrease in bone formation at the tissue level during human postnatal calvarial osteogenesis is associated with decreased cell growth and increased differentiation of osteoblastic cells at the cellular level. Accordingly, human calvaria cells isolated from young individuals with high bone formation activity in vivo and high growth of osteoblastic cells in vitro had the ability to form bone-like structures in vitro.

The age-related changes in calvarial osteogenesis were evaluated by measuring histomorphometric indices of bone formation at different postnatal ages. We found that bone formation was very active at 3–4 months of age in normal calvarial bone, consistent with a high rate of

TABLE I. Variable Expression of Bone Matrix Protein in the Calcified Matrix and Osteoblasts During Postnatal Human Calvaria Osteogenesis*

Matrix proteins	Postnatal age (months)							
	4	5	6	7	10	14	17	30
Type I collagen	+	+	±	±	+	+	+	+
Osteonectin	−	±	−	−	±	±	+	−
BSP	±	±	−	−	−	±	±	+

*Human calvarial sutures were embedded in paraffin, immunohistochemistry was performed using specific antibodies, and the immunostaining was evaluated in comparison with control sections incubated without primary antibody. The staining for bone matrix proteins was scored as negative (−), weakly positive (±), and intense staining (+). Type I collagen was more expressed than osteonectin and bone sialoprotein (BSP), and the staining varied between individuals, with no apparent relation with age.

Fig. 4. Microphotographs of human osteoblastic cells derived from normal calvaria showing expression of alkaline phosphatase activity (**a**) and bone matrix proteins (**c–f**) revealed by immunocytochemistry. About 50% of the cells expressed alkaline phosphatase (a) and 10–30% of the cells stained positively for collagen type I (c), osteocalcin (d), osteonectin (e), or biglycan (f), compared to unlabeled control cells incubated with non-immune serum (b). (Magnification: ×250).

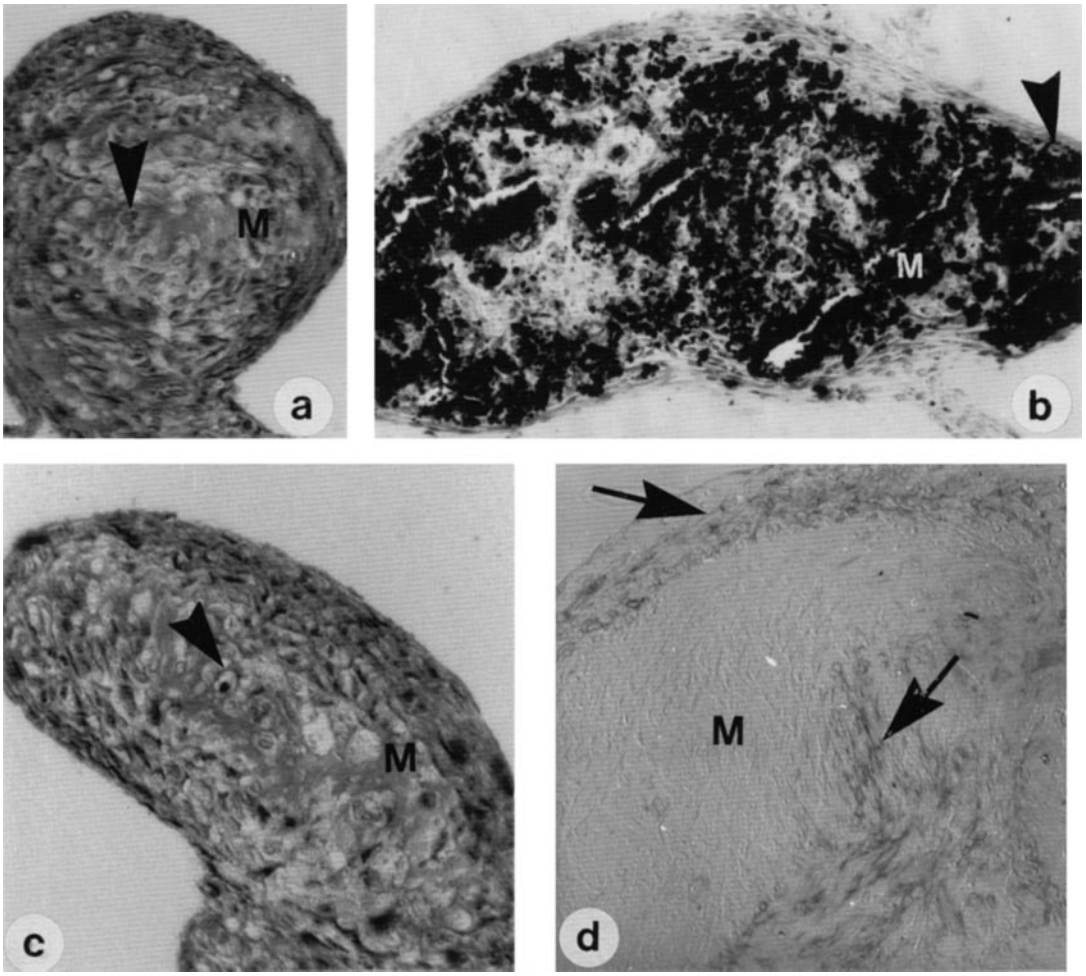

Fig. 5. Histological appearance of nodular structures formed in vitro by human calvaria osteoblastic cells cultured for 3 weeks in the presence of ascorbic acid and β-glycerophosphate. Five µm-thick sections were stained with toluidine blue (**a, c**) or von Kossa (**b**), or were unstained and cytochemically reacted for alkaline phosphatase activity (**d**). The calvaria cells formed three-dimensional small (a) or large nodular structures (c) at 21 days of culture. The nodular structure showed a woven bone-like appearance with osteocyte-like cells (arrows) embedded in the matrix (M) (a, c). Numerous cells surrounding the matrix (M) showed alkaline phosphatase activity (arrows) (d). The bone-like structures became progressively mineralized as shown by von Kossa staining (b). Magnification: b: ×125; a, c, d: ×250.

osteogenesis in the postnatal life. The extent of bone forming sites declined progressively with age, indicating that the osteogenic activity decreased during calvaria ossification in the first 18 months of age. This is in accordance with the reported decrease in trabecular bone formation with age in children [Fedarko et al., 1992]. In the absence of dynamic markers of bone matrix formation, we could not identify whether the activity of human calvaria osteoblasts declined with age. We used an immunohistochemical analysis to evaluate the age-related changes in bone matrix proteins synthesized by osteoblasts during postnatal calvarial osteogenesis. The immuno-histochemical analysis at different ages showed that type I collagen, bone sialoprotein, and osteonectin were expressed in osteoblasts and the matrix, as found in adult human trabecular bone [Bianco et al., 1990, 1991; Fisher, 1995]. We found no apparent change with age in the expression of these bone matrix proteins during postnatal calvarial osteogenesis. This does not exclude the possibility that the rate of production of these proteins changed with time since the expression of bone matrix proteins decreases with age in human trabecular bone cells [Fedarko et al., 1992].

While the metabolism and in vitro behavior of human endosteal osteoblastic cells in relation to age [Fedarko et al., 1992] and bone

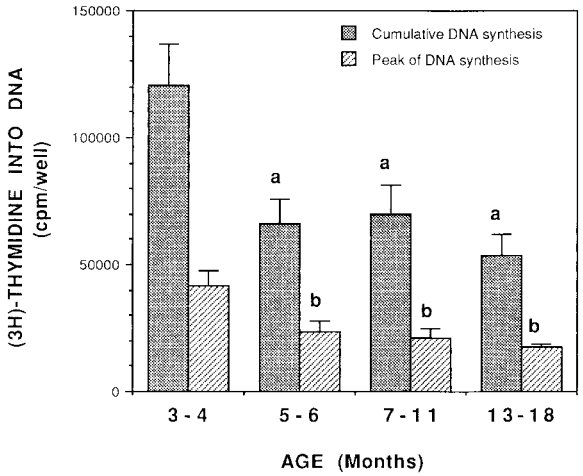

Fig. 6. Age-related decrease in the proliferation of human calvaria osteoblastic cells during postnatal osteogenesis (n = 36). The mean DNA synthesis was higher at 3–4 months of age compared to other age groups. Cell growth declined from 3–4 months to 13–18 months of age. The data are the mean ± SEM of 4–11 cultures per time-point. Each value is the mean of 3–4 cultures. **a, b:** A significant difference with the respective value at 3–4 months of age ($P < 0.05$).

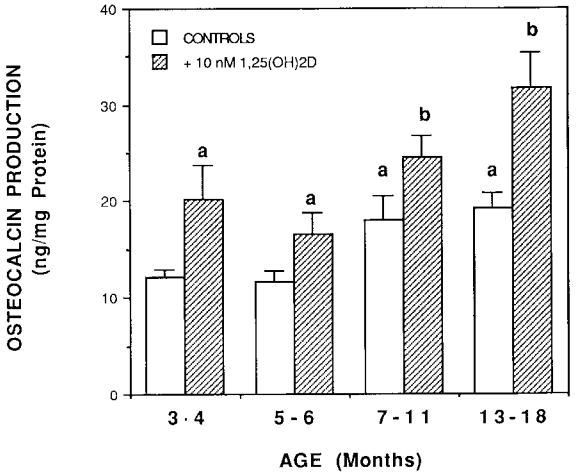

Fig. 8. Age-related increase in the mean osteocalcin production by human calvaria osteoblastic cells during postnatal osteogenesis (n = 23) in basal conditions and after stimulation with $1,25(OH)_2D$ (10 nM, 48 h). The data are the mean ± SEM of 5–6 values per time point. Each value is the mean of 3–4 cultures. **a:** A significant difference with values at 3–4 months of age. **b:** A significant difference with paired controls ($P < 0.05$).

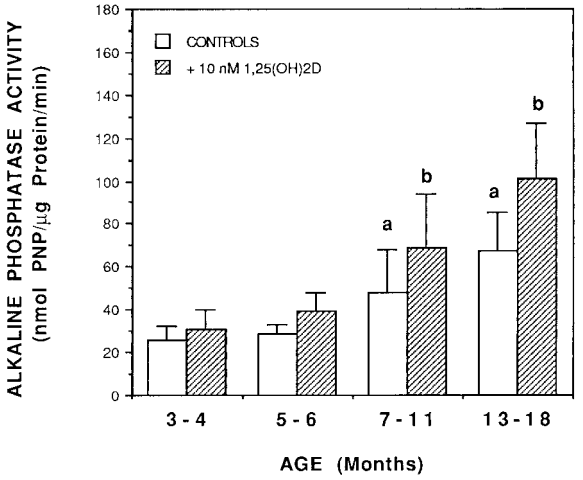

Fig. 7. Age-related increase in the mean alkaline phosphatase activity in human calvaria osteoblastic cells during postnatal osteogenesis (n = 30) in basal conditions and after stimulation with $1,25(OH)_2D$ (10 nM, 48 h). The data are the mean ± SEM of 5–11 values per time point. Each value is the mean of 3–4 cultures. **a:** A significant difference with values at 3–4 months of age. **b:** A significant difference with paired controls ($P < 0.05$).

diseases [Marie et al., 1991; Marie, 1994] have been well studied, the phenotype of human calvaria bone cells has not been previously characterized. We have, therefore, analyzed the phenotype of cells derived from human calvaria and show that cells isolated from normal human calvaria express characteristics of the osteoblast phenotype. This was documented by the high alkaline phosphatase activity, osteocalcin production, which are early and late markers of the osteoblast phenotype, respectively [Rodan and Rodan, 1990], response to calciotropic hormones and osteogenesis in vitro for cells from younger individuals. These characteristics of human calvaria-derived cells are similar to those of human trabecular bone cells, with the important exception that the latter have not been shown to form bone nodular structures in vitro. Indeed, although human osteoblastic cells derived from the endosteum or from the marrow stroma show several osteoblastic characteristics [Robey and Termine, 1985; Beresford et al., 1986; Marie et al., 1989; Cheng et al., 1994], human trabecular bone cells or marrow stroma-derived cells do not readily form bone-like calcified nodules in vitro [Beresford et al., 1993; Cheng et al., 1994]. In contrast, we found that human calvaria osteoblastic cells derived from young individuals have the ability to form bone-like nodular structures when cultured in conditions that facilitate the induction of osteogenesis in vitro [Ecarot-Charrier et al., 1983; Nefussi et al., 1985; Bellows et al., 1986; Lomri et al., 1988; Owen et al., 1990; Modrowski and Marie, 1993]. The mineralized bone-like nodular structures formed by human calvaria osteoblastic cells resembles the calcified nodules formed by rat and mouse calvaria cells in vitro [Ecarot-Charrier et al., 1983;

Nefussi et al., 1985; Bellows et al., 1986; Owen et al., 1990], indicating that these human calvarial cells are osteogenic in vitro.

Only human calvarial cells isolated from younger subjects had the ability to form nodular structures, suggesting age-related differences in phenotypic characteristics of the isolated cells. We have thus determined the age-related changes in human calvaria cell proliferation and differentiation during postnatal osteogenesis. The analysis of DNA synthesis showed that cell growth was high at 3–4 months of age and decreased with age during the development of postnatal osteogenesis. Since only a fraction of the cell population studied exhibited markers of the osteoblast phenotype in vitro in basal conditions, it is likely that the age-related change in cell proliferation reflected the growth of osteoblast precursors rather than that of more differentiated cells, as found in the rat calvaria model [Owen et al., 1989; Stein et al., 1990]. Thus, the ability to form bone-like structures by human osteoblastic cells in vitro correlates with the high bone formation activity in vivo and the high growth of osteoblast precursors in vitro, which is consistent with our previous data in trabecular bone showing that the activity of bone formation is mainly related to the osteoblastic cell growth potential [Marie et al., 1991; Marie, 1994, 1995].

In contrast to the age-related decline in osteoblastic cell growth, we found that alkaline phosphatase rose during postnatal calvarial osteogenesis, suggesting increased differentiation of osteoblastic cells. The age-related increase in osteocalcin production, a marker of mature osteoblasts [Rodan and Rodan, 1990; Turksen et al., 1992], further indicates that osteoblast differentiation increases progressively during calvarial osteogenesis. These data suggest that the age-related decline in human osteoblastic cell growth is followed by osteoblast differentiation during the progression of postnatal calvaria osteogenesis. This inverse relationship between cell proliferation and differentiation in human calvaria cells is consistent with the decrease in osteoblastic cell growth followed by progressive maturation of osteoblasts during the development of osteogenesis in neonatal rat calvaria in vitro [Owen et al., 1990; Stein et al., 1989, 1990] and in vivo [Machwate et al., 1995].

The results of the present study show that the age-related decline in bone formation during human postnatal calvaria osteogenesis is associated with decreased osteoblastic cell growth, followed by increased cell differentiation. Human osteoblast precursor cells with high growth potential have the ability to form bone-like structures in vitro, providing a new in vitro model to study human osteoblast differentiation and osteogenesis. This new model will be useful to analyze the cellular and molecular mechanisms involved in normal calvaria osteogenesis and in abnormal bone formation in humans, such as in premature cranial ossification or craniosynostosis [Cohen, 1993; Marchac and Renier, 1982].

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