Journal of Cellular Biochemistry 75:22–35 (1999)

Development of the Osteoblast Phenotype in Primary Human Osteoblasts in Culture: Comparison With Rat Calvarial Cells in Osteoblast Differentiation

Heide Siggelkow,[1]* Katja Rebenstorff,[1] Wiebke Kurre,[1] Christopher Niedhart,[1] Iris Engel,[1] Hiltrud Schulz,[1] Michael J. Atkinson,[2] and Michael Hüfner[1]

[1]Department for Gastroenterology and Endokrinology, Georg-August-University, D-37075 Göttingen, Germany
[2]Institute for Pathology, GSF München, D-85758 Oberschleißheim, Germany

Abstract In rat osteoblast-like cells, a time-dependent sequence of growth and differentiation-dependent genes has been identified and a model of osteoblast differentiation in culture suggested. We investigated the expression of the bone matrix-associated proteins osteonectin and procollagen I and of the bone cell phenotype-related proteins alkaline phosphatase and osteocalcin during cell culture in primary human osteoblast like cells. Primary human explant cultures from nine young healthy donors were established under highly standardized conditions. Cells in the second passage were analyzed on different days from day 1 to 32, comparing cells growing under the influence of ascorbate with controls. Gene expression was determined by Northern blot analysis or polymerase chain reaction. Osteocalcin expression was also investigated after 1,25-$(OH)_2D_3$ stimulation. On the protein level, newly synthesized collagen I, alkaline phosphatase activity, and secretion of osteocalcin were analyzed at all time points. On comparing our findings to the pattern of gene expression suggested for the rat calvarial osteoblast system, we found a similar developmental sequence for the so-called "proliferation" as well as a similar, but lengthened, sequence for the "matrix maturation stage." During "matrix maturation," we found an ongoing proliferation despite increased alkaline phosphatase and decreased procollagen I gene expression. Our study, therefore, shows that in pHOB the gene expression profile proceeded to the "matrix maturation stage," as defined by Owen and colleagues, independent of ongoing proliferation. We were unable to observe the mineralization period as demonstrated by the missing increase of osteocalcin expression and lack of nodule formation in our human osteoblast model. In contrast to the rat system, we found a proliferation stimulating influence of ascorbate, suggesting species-specific differences in response to differentiation factors. From these data, we conclude that general considerations on physiology and pathophysiology of bone cell differentiation have to be confirmed in the human osteoblastic cell system. J. Cell Biochem. 75:22–35, 1999. © 1999 Wiley-Liss, Inc.

Key words: human primary osteoblasts; differentiation stages; alkaline phosphatase; osteocalcin; osteonectin; collagen I; time in culture; regulation

During the last few years, increasing amounts of information on characteristics of osteoblast differentiation have been accumulated through in vitro and in vivo systems [Gerstenfeld et al., 1987; Nefussi et al., 1997; Owen et al., 1990; Quarles et al., 1992; Siggelkow et al., 1998d, Choi, 1996]. Osteoblast differentiation includes a phase of cell proliferation, followed by coordinated expression of bone-related proteins [Stein and Lian, 1993]. Corresponding to the in vitro results, this expression pattern for bone-related proteins has also been confirmed in in vivo systems [de Pollak et al., 1997; Dodds et al., 1994; Mundlos, 1994; Nefussi et al., 1997; Nomura et al., 1988; Weinreb et al., 1990; Yoon et al., 1987]. During proliferation, this sequence is characterized by the temporal expression of cell cycle or growth-related genes (histone, c-*myc*, and c-*fos*) and by genes of extracellular matrix proteins (procollagen I, transforming growth factor-β [TGF-β], fibronectin). This is followed by a phase of matrix maturation characterized by maximal expression of alkaline phosphatase (AP). At the beginning of mineralization, genes for proteins such as osteocalcin, bone sialoprotein, and osteopontin are expressed [Stein and Lian, 1993]. This so-called

Grant sponsor: Bundesministerium für Forschung und Technologie (BMFT); Grant number: 01 KM 9301/2.

*Correspondence to: H. Siggelkow, Department of Gastroenterology and Endocrinology, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany.

Received 16 November 1998; Accepted 17 February 1999

© 1999 Wiley-Liss, Inc.

developmental sequence can be characterized in three stages: (1) rapid proliferation, (2) matrix maturation, and (3) mineralization.

This sequence was most extensively studied in the rat calvarial cell system [Aronow et al., 1990; Collin et al., 1992; Nefussi et al., 1997; Owen, 1985; Owen et al., 1990, 1991; Pockwinse et al., 1995], but it was also studied in chicken calvarial osteoblasts [Gerstenfeld et al., 1987] and MC3T3-E1 cells [Quarles et al., 1992]. Although similarities in the expression of several osteoblast-regulated genes have been found, species differences have also become evident [Cheng et al., 1994; Choi et al., 1996]. Information on the differentiation of human osteoblasts is sparse. Initial studies in developing human bone appear to indicate an analogous process [Dodds et al., 1994; Mundlos, 1994]. The expression sequence was partly reproduced in human calvarial cells [de Pollak et al., 1997], but differences in the rat cell system were seen in immortalized human fetal osteoblasts overexpressing the estrogen receptor [Robinson et al., 1997] and in human osteosarcoma cells [Siggelkow et al., 1998d]. To date, the expression pattern of bone-related proteins observed in other species has not been investigated in primary human osteoblast cells from adult spongiosa and therefore remains to be determined.

For the study of human primary osteoblasts, several cell models are in use, including "pure" explant cultures [Beresford et al., 1984], cultures from collagenase digested bone explants [Gehron Robey and Termine, 1985], as well as models originating from bone marrow representing human osteoblast precursor cells [Cheng et al., 1994; Kassem et al., 1991]. The cells obtained by these methods synthesized all the major noncollagenous proteins and predominantly collagen I. However, mineralization occurred only when cells were cultured with glucocorticoids, β-glycerophosphate (bGP), and ascorbate [Gundle and Beresford, 1995]. Nodule formation, regularly observed in rat [Bellows et al., 1986; Nefussi et al., 1985; Tenenbaum and Heersche, 1982], chicken [Gerstenfeld et al., 1988], and human calvarial cells isolated from newborn human subjects [de Pollak et al., 1997], could not be reproduced in primary human osteoblasts from adult spongiosa in vitro [Ashton et al., 1985; Gundle and Beresford, 1995]. Nevertheless, the explant system is thought to serve as an excellent culture model to investigate human physiology and pathology [Gundle and Beresford, 1995].

The purpose of this study was to determine the expression pattern of the bone-related proteins during osteoblast differentiation in primary human osteoblasts from spongiosa of healthy adults and to compare it with the known data of the rat system [Owen et al., 1990]. We studied the matrix-associated genes procollagen I and osteonectin (ON), as well as gene expression of the osteoblast-characteristic proteins alkaline phosphatase (AP) and osteocalcin (OC) under baseline conditions and under the influence of ascorbate. We also determined the activity of the alkaline phosphatase enzyme and the secretion of osteocalcin and collagen I proteins.

MATERIALS AND METHODS

Reagents

All cell culture media and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Cell culture disposables were purchased from Nunc (Roskilde, Denmark) or Greiner (Solingen, Germany) and medium supplements (antibiotics, glutamine) from GIBCO-BRL (Eggenstein, Germany). Standard laboratory reagents were purchased from Sigma Chemical Company (Munich, Germany), if not noted otherwise.

Patients

Bone specimens were obtained from the iliac crest of 9 men aged 19–54 years (median age 26 years, mean 30.2 ± 3.4 years) undergoing corrective surgery after fracture. None of the patients showed any signs of bone or joint disease or autoimmune disorder. Informed consent was obtained from all patients. The study was approved by the local ethical committee.

Bone Preparation and Cell Culture

Primary human bone cell cultures were prepared from bone specimen as previously described [Auf'm Kolk et al., 1985; Beresford et al., 1984; Siggelkow et al., 1998a]. Trabecular bone fragments from the iliac crest were cut into pieces, thoroughly rinsed in phosphate-buffered saline (PBS), and kept in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, glutamine (58.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures

were initiated within 3 h and fed twice a week. Cells from the explants grown on the culture dish were released from the surface with 0.25% trypsin after 14–21 days, divided into two 75-cm^2 culture flasks, maintained in medium at 37°C in a humidified 95% air/5% CO_2 atmosphere and defined as first passage cells. Trypan blue was used to correct for dead cells. After growth to 100% confluence, the cells were released from the culture dish as described above, cell number was determined by hemocytometer and cells were plated at a density of 3×10^5 cells/dish (4×10^3 cells/cm^2). For analysis of differentiation, ascorbate (50 μg/ml) was added at time of confluence (day 10). Cells were analyzed in the second passage every 4 days at day 1 until day 32. Cells were always fed 24 h before analysis. One charge of serum was used for all experiments. All cells were regularly tested for mycoplasma contamination by polymerase chain reaction (PCR) (Mycoplasma PCR Primer Set, Stratagene, Heidelberg, Germany).

Histochemical Analysis

Although the cells were cultured without the addition of β-glycerophosphate (bGP) and nodule formation was not expected, we monitored for calcium phosphate salt formation using Von Kossa staining of ethanol-fixed multilayers. Ponceau acid fuchsin was used as a counterstain. Before light microscopy, cells were serially washed with 1% acetic acid and absolute ethanol.

Analysis of Total Protein

For the determination of total protein and alkaline phosphatase (AP), cells were lysed in distilled water by repeated freeze-thawing and sonication (Ultrasonics W 185F, 40–60 W, 3 × 30 s). The lysate was clarified by centrifugation (10 min at 10,000*g*) and the soluble protein fraction quantified using the BioRad protein assay with an albumin standard (BioRad, München, Germany).

DNA Assay

DNA was determined in the cell lysate by using the Hoechst fluorescent dye (Hoechst 33258) [Rago et al., 1990]. Fluorescence was quantified with a Cytofluor 2300 plate reader (Millipore, Eschborn, Germany).

Analysis of Alkaline Phosphatase and Procollagen I

AP activity was assayed in cell lysates by determining the release of p-nitrophenol from p-nitrophenyl phosphate at 37°C, pH 10.5. The substrate solution contained 8 mM p-nitrophenyl phosphate in 0.5 M AMP-buffer (2-amino-2-methyl-1-propanol), pH 10.5, supplemented with 0.2 mM $MgCl_2$. After 10 min at 37°C, the reaction (volume 110 μl) was stopped with 50 μl 1 N sodium hydroxide. AP activities were measured in duplicate and are reported as nmol/10^5 cells per minute incubation time (modified from Boyan et al. [1989]). The secreted C-terminal peptide of procollagen I (PICP) was measured in culture supernatants harvested 24 h after feeding. Collagen type I content was calculated from the C-terminal propeptide concentration [Jukkola et al., 1991; Melkko et al., 1990] measured in duplicate samples by enzyme-linked immunosorbent assay (ELISA) (Metra Biosystems Palo Alto, CA). Values were adjusted for background levels of PICP in culture medium.

Determination of Osteocalcin Production

pHOB cells from eight donors were grown to confluence (day 10) in culture medium and then switched to incubation medium containing DMEM with 1% bovine serum albumin (BSA) with 5×10^{-8} M 1,25-$(OH)_2$-D_3 or solvent (ethanol $<0.01\%$) for 48 h at 37°C. The medium was then collected and stored at −70°C until assay for osteocalcin. Cell numbers were determined at each time point by hemocytometer counting. Determination of osteocalcin was performed in duplicate with an immunoradiometric assay (Nichols, Bad Nauheim, Germany); the detection range of the kit was 0.9–267 ng/ml. Values were corrected for interfering substances present in the culture medium.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from cells by a phenol/chloroform-extraction method [Chomczynski and Sacchi, 1987]. For Northern blot analysis, 10 μg per lane of total cellular RNA was separated electrophoretically in a 1% agarose gel (2% formaldehyde, 0.3 μg/ml ethidium bromide), blotted to nylon membranes (Zeta Probe, BioRad, München, Germany) by capillary diffusion, and immobilized by ultraviolet (UV) crosslinking (Stratalinker, Stratagene, Heidelberg). ^{32}P-labeled cDNA probes were synthesized by

random priming (Stratagene, Heidelberg) [Feinberg and Vogelstein, 1983]. Prehybridization and hybridization were performed in 50% formamide, 4 × SSPE, 5 × Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 500 μg/ml salmon sperm DNA at 42°C for 24 h. For hybridization approximately 10^6 cpm/ml of ^{32}P-labeled probe was added. For labeling of ribosomal RNA, the 5′-end-labeling kit (Boehringer Mannheim, Germany) was used; during hybridization, 20 μg of nonradioactive oligonucleotides was added. After hybridization, blots were washed for 2× 30 min at room temperature in 2× SSC/0.1% SDS and 5–30 min at 55°C (rat cDNA probes) or 63°C (human cDNA probes) in 0.1× SSC/0.1% SDS. Autoradiographs were quantified by laser densitometry (Biometra, Göttingen, Germany). End-labeling of 18S and 28S ribosomal RNA was used to ensure equal loading of RNA in all samples [Barbu and Dautry, 1989]. Gene expression was standardized to ribosomal RNA and calculated in arbitrary OD units with the maximum absorbance on each blot set at 100 U. Values are therefore expressed in relation to the maximal absorbance of each transcript. Values represent the mean of nine different cultures, if not stated otherwise. Two sets of Northern blots were rehybridized without stripping for each experiment.

cDNA Probes

Procollagen type I expression was measured using the rat proα(I) collagen cDNA clone pHCAL1U [Vuorio et al., 1987] detecting the 5.9 and 7.2 transcripts. The alkaline phosphatase human cDNA probe was a 2.5-kb insert of pAT153 [Weiss et al., 1986] with the transcript size of 2.6 kb. Osteocalcin expression was analyzed using the human 1.2-kb *Sac*I fragment of SP 65 [Celeste et al., 1986] with a transcript size of 0.6 kb. The human osteonectin gene expression was measured with the 1.2-kb cDNA insert of clone HHCH67 [Adams et al., 1991] detecting the 2.2 transcript. A 26-bp 28S-oligonucleotide probe (Pharmacia, Germany) was used to detect the 4.7 transcript of ribosomal RNA [Barbu and Dautry, 1989].

RT-PCR

cDNA was synthesized from 1 μg of total RNA in a 40-μl reaction mixture containing 1× reverse transcriptase buffer, consisting of 5× = 15 mM $MgCl_2$, 375 mM KCl, 250 mM Tris-HCl (pH 8.3), 50 mM DTT), dCTP, dGTP, dATP, and dTTP each at 2 mM (Boehringer Mannheim), 40 U of RNAse inhibitor (Boehringer Mannheim), 400 U of M-MLV reverse transcriptase (Gibco, Germany), and 80 pmol of poly-dT15 primer (Boehringer Mannheim). Reaction times were at least 1 h at 38°C and 10 min at 72°C. Aliquots (2.5%) of the total cDNA were amplified in each PCR in a 15-μl reaction mixture that contained 10 pmol of 5′ and 3′ primer, 1× = PCR buffer, consisting of 10 × = 500 mM KCl (pH 8.3), 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, and dCTP, dGTP, dATP, and dTTP each at 0.2 mM and 0.5 U of *Taq* polymerase (Boehringer Mannheim). Each cDNA sample was run in duplicate for every PCR. Amplifications were performed in a Primus PCR-Thermo-Cycler (MWG-Biotech, Germany) for 30 cycles after an initial denaturation at 94°C for 2 min. The after reaction profile was used: 94°C for 30 s, 55°C for 2 min, and 72°C for 2 min. For the amplification reaction of mRNA of osteocalcin and lipoprotein lipase (LPL), the PCR primer sequences were used as described [Rickard et al., 1996]. Reaction products were analyzed by electrophoresis of 15-μl samples in 1.5% agarose gels. The amplified DNA fragments were visualized by ethidium bromide staining.

Fluorescence Activated Cell Scanning

Fluorescence activated cell scanning (FACS) analysis was conducted as described [Siggelkow et al., 1998b]. In short, after trypsinization 100,000 cells per sample were pipetted into a well of a 96-well microplate (V-shaped bottom). Addition of the protein-specific primary antibody (1:50) was followed by incubation for 20 min at room temperature. After two repeated washings with PBS washing buffer, the cells were incubated with the preabsorbed secondary FITC-conjugated antibody for 60 min at room temperature and after repeated washings resuspended in 400 μl PBS with 1% formaldehyde. To detect any changes in cell size (forward cell scatter, FSC) or cell granularity (sideward cell scatter, SSC) caused by antibodies or chemicals, one control was always incubated with PBS washing buffer only. To test for nonspecific binding of the two different FITC-labeled secondary antibodies (goat anti-rabbit and goat anti-mouse; Sigma), cells were preabsorbed with human sera and then incubated with secondary antibodies only. Any staining in these samples was defined as nonspecific binding. Each analysis was done in triplicate and

results are mean values ± standard error of the mean (±SEM) A FACS 81553 (Becton-Dickinson Immunocytochemistry Systems, Mountain View, CA) was used for analysis of fluorescent cells based on the reading of 5,000 events (any particle detectable by FACS) per sample. In all experiments, the FACS 81553 (gain, amplifier, and photo multiplier voltage) was kept at a constant setting. Analysis was done with FACS Research Software Version 2.1 3/89.

Permeabilization of pHOB for FACS

Permeabilization with saponin permits detection of intracellular proteins with FACS [Jung et al., 1993]. Freshly trypsinized pHOB were incubated in an ice-cold PBS buffer with 4% formaldehyde for 10 min at 4°C. After centrifugation for 6 min at 300*g*, the pellet was resuspended in 0.1% saponin (Sigma), which was added to all the different preparation steps for FACS thereafter. Incubation with the antibodies was conducted as described above. Before analysis in FACS, cells were resuspended in 400 μl PBS with 1% formaldehyde.

Antibodies

To detect the intracellular protein osteocalcin, the polyclonal anti-human osteocalcin antibody (Biogenesis, Poole, England) was used. Mouse anti-human thyroglobulin [Schulz et al., 1989] served as the negative control. The FITC-conjugated secondary antibodies used were goat anti-rabbit IgG and goat anti-mouse IgG (both Sigma).

Statistical Analysis

All values are expressed as mean ±SEM. Statistical testing was carried out using Win-Stat-Statistics or Statistica 6.0 for Windows, the Wilcoxon nonparametric test was used if not noted otherwise. Significant differences ($P < 0.05$, $P < 0.01$, $P < 0.001$) are marked with *, **, and ***, respectively.

RESULTS

Morphological Appearance

We chose the method of Beresford et al. [1984] for isolating the primary human osteoblast-like cells. This method of isolation was shown to produce cells able to mineralize only when cultured with ascorbate, bGP, and glucocorticoids, whereas nodule formation was only seen in diffusion chambers implanted into athymic mice [Gotoh et al., 1995]. We decided to investigate the cells under baseline conditions and under the influence of ascorbate, which is known to be a prerequisite for matrix synthesis [Aronow et al., 1990; Franceschi et al., 1994; Gerstenfeld et al., 1988; Owen et al., 1990]. Figure 1 demonstrates the morphological appearance of pHOB during culture. The cells showed the typical fibroblast-like appearance during early proliferation (Fig. 1A) and became polygonal with multiple extrusions later in culture (Fig. 1B). Approaching confluence the shape took on an elongated and thickened appearance (Fig. 1C). After confluence, the cells became more elongated, closely packed and began to multilayer (Fig. 1D). With van Kossa staining, no nodule formation was seen (data not shown). Only when using dexamethasone, daily addition of ascorbate and bGP, the cells showed calcium phosphate deposition demonstrable with energy-disperse x-ray microanalysis (data not shown). Therefore, the development of the human primary culture seems to be different from rat calvarial cells showing mineralization of primary cultures from day 19 when cultured with ascorbate and bGP added at confluence. In rat calvarial cells, nodule formation correlated with plating density [Aronow et al., 1990; Bellows et al., 1986; Tenenbaum and Heersche, 1982] and decreased when cells were subcultivated [Aronow et al., 1990]. We had to use second-passage cells with one-fifth lower seeding density, owing to the limited availability of cells from disease-free human bone explants. To overcome another limitation, the difficulty in obtaining reproducible cultures due to high interpatient variations, we used only material from young (median age 26 years, mean 30.2 ± 3.4 years), otherwise healthy men operated on for fracture repair. Hence, the differences described for cell cultures obtained from patients of different age groups [Battmann et al., 1997; Evans et al., 1990] could be avoided. Primary cultures were established only from material originating from the iliac crest because differences in cells from different harvest sites have been described [Kasperk et al., 1995]. With these highly standardized culture conditions and the use of 5–10 cultures, depending on the individual experiment, we were able to reduce the variation in results to a level until now obtained only for human osteosarcoma cells [Siggelkow et al., 1995].

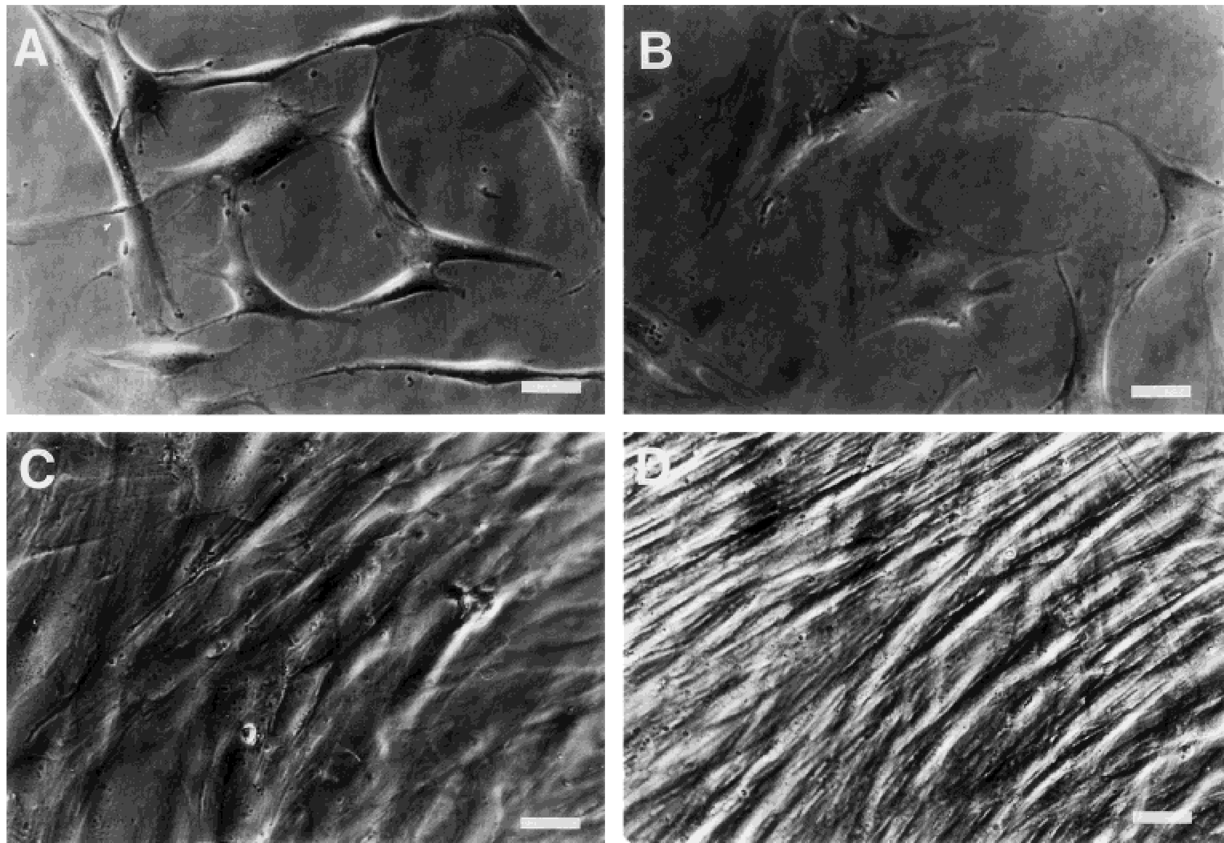

Fig. 1. Representative light-field microscopy of primary human osteoblasts cultured in the presence of 50 μg/ml ascorbate added after day 10, at different times in culture. Photomicrographs from day 3 (**A:** 200-fold, Scale bar = 40 μm), 5 (**B:** 200-fold, Scale bar = 40 μm), day 10 (**C:** 200-fold, Scale bar = 40 μm), and day 32 (**D:** 200-fold, Scale bar = 40 μm).

Growth Characteristics of pHOB

We plated our cells in the second passage at 4,000 cells/cm^2 to reach confluence at around day 10. In first passage rat calvarial cells confluence was reached on day 7 with a plating density of 5,000 cells/cm^2 [Owen et al., 1990]. Chicken embryo calvarial cells, plated with 20,000 cells/cm^2 in the first passage, were confluent between day 6 and 8 [Gerstenfeld et al., 1987] and MC3T3-E1- cells showed confluence before day 5 (20,000 cells/cm^2) [Choi et al., 1996]. Regarding the human cell system, confluence was reached at day 6 when plated with 10,000 cells/cm^2 in immortalized fetal osteoblasts [Robinson et al., 1997], but in human calvarial cells the plating density was much higher (≤1,000,000 cells/cm^2), so that confluence must have been reached much earlier [de Pollak et al., 1997]. A survey of plating densities in the different culture systems investigated suggests that a comparison between different species might be difficult, mainly due to the variation of experimental conditions. The plating density used in our experiments was relatively close to the cell number used in rat calvarial cells.

In our system, cell numbers increased 4-fold from day 1 to confluence (day 10) but thereafter only doubled until day 32 (Fig. 2A). The use of ascorbate increased cell proliferation to a 2.8-fold higher cell number at day 32 ($P < 0.01$, Fig. 2A). The values for total protein and DNA correlated directly with cell number, and the latter was later used to standardise the characteristic proteins (Spearman rank-order correlation: $r = 0.96$, $P < 0.001$ and $r = 0.99$, $P < 0.001$, respectively). Proliferation of control cells decreased during time in culture, whereas, under the influence of ascorbate, proliferation was initially accelerated, slowing down at the beginning of day 25, compared with the preconfluent stage. Surprisingly, there was a further significant increase of cell numbers and DNA content from day 28 to 32, suggesting ongoing proliferation

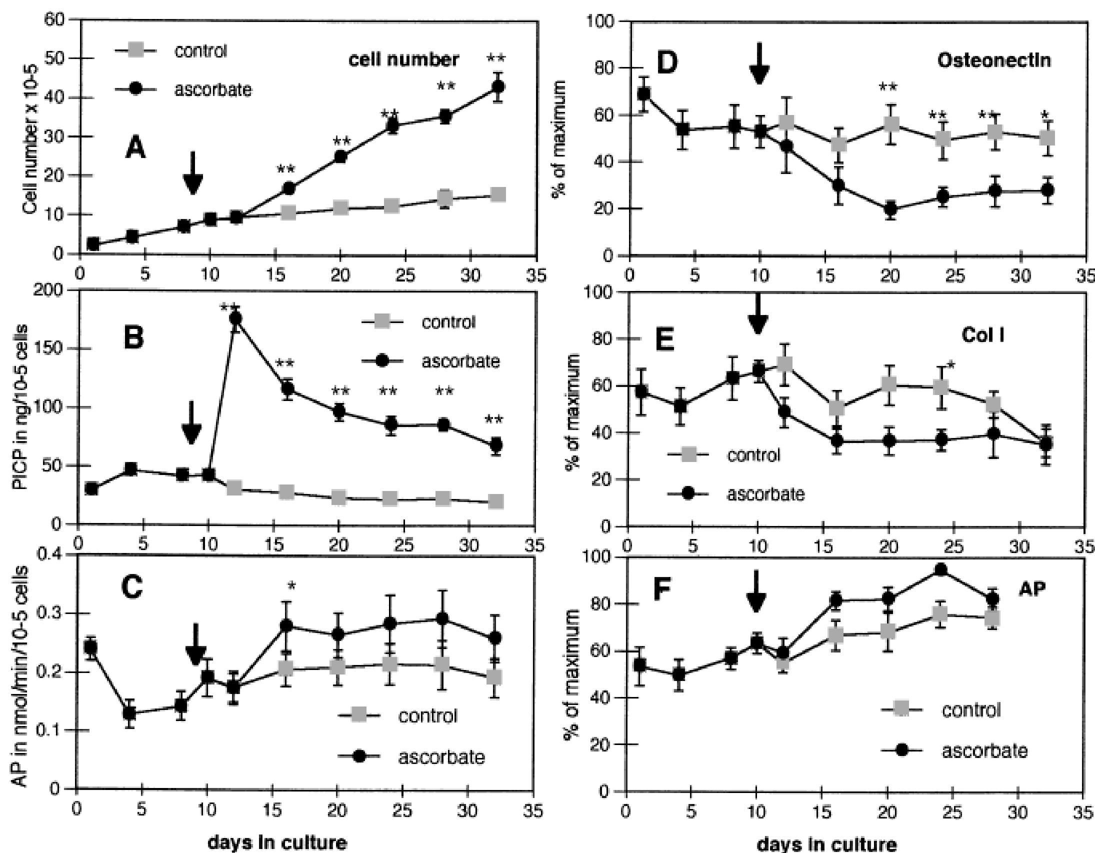

Fig. 2. Proliferation, protein secretion, and gene expression during in vitro maturation of human primary osteoblasts (pHOB) in culture. Cellular RNA from nine independent experiments was isolated at the days indicated and assayed by Northern blot analysis. Left, proliferation **(A)**, procollagen I (PICP) secretion **(B)**, and AP activity **(C)** under the influence of 50 µg/ml ascorbate added at confluence compared with control during 32 days in culture (mean values ±SEM. for nine cultures). The significant differences between ascorbate and control cultures are marked with *($P < 0.05$) or **($P < 0.01$), (Wilcoxon signed rank test). Right, the relative gene expression in percentage of maximum expression (mean values ±SEM). Expression was quantified by laser densitometry. Results were plotted against the maximum expression of each transcript and standardized to 18/28S RNA for nine (**D**, osteonectin; **E**, procollagen, Col I) or five (**F**, alkaline phosphatase, AP) cultures.

at this time point (Fig. 2A). By contrast, in rat calvarial cells, ascorbate inhibited cell proliferation (as shown by an accentuated plateauing at day 14 in culture), whereas without ascorbate cells exhibited continuous growth [Owen et al., 1990]. The proliferation stimulating effect of ascorbate demonstrated in our system was also seen in MC3T3-E1 [Dean et al., 1994; Harada et al., 1991], and in rat calvarial cells after confluence [Spindler et al., 1989]. However, an inhibiting influence [Owen et al., 1990; Sugimoto et al., 1986], or no effect has also been described in other cell systems [Choong et al., 1993; Quarles et al., 1992] and was attributed to species differences. To date, we have no explanation for the differing effects of ascorbate on rat and human cells.

Day 1 in culture

Although we demonstrate the results of protein synthesis and gene expression on day 1, we are convinced that the cells were not yet equilibrated and that they still exhibited some traits characteristic of the end of the first passage. This was also seen in other systems [Sautier et al., 1993] and interpreted as transient dedifferentiation of cells in an active proliferating stage [Nefussi et al., 1997]. Therefore, we are hesitant to speculate on the results of this time point.

Expression of Bone-Related Proteins Procollagen I and AP During Cell Differentiation

During the preconfluent growth phase we saw a significant 1.5-fold increase in procolla-

gen synthesis as measured by PICP secretion in the supernatant ($P < 0.05$, Figs. 2B, 3C). After confluence, PICP secretion decreased from day 10 to 12 ($P < 0.05$) and, after day 16 was significantly lower compared with day 1 ($P < 0.05$). Secretion was 43% of maximum levels at day 32 in culture. Ascorbate stimulated the PICP secretion 5.8-fold at day 12 when added at confluence. This stimulating effect decreased with further culture time down to 3.2-fold at day 32 compared with control cultures ($P < 0.05$, Fig. 2B). We assume that the pronounced effect at confluence is due to stimulated hydroxylation, secretion, and processing of preformed type I procollagen components. Franceschi et al. [1994], who studied collagen metabolism intensively in chicken osteoblasts, saw only minor changes in procollagen synthesis and gene expression but a sharp increase in hydroxylation after the addition of ascorbate. In our ascorbate stimulated cultures, the PICP secretion decreased during time in culture to 36% of maximum from day 12 to 32 ($P < 0.01$, Fig. 3D). Therefore, the sequence of expression of baseline and stimulated PICP level differed only in absolute values, showing both a maximum expression at confluence and a decrease thereafter (Fig. 2B). It has been shown that synthesis of new collagen protein increased during proliferation in chicken osteoblasts but decreased thereafter, whereas collagen in the matrix increased further [Gerstenfeld et al., 1988]. In our experiments procollagen secretion was measured with a PICP-specific antibody, which binds to the intact procollagen I molecule, as well as to the free C-terminal propeptide, which is split off during collagen I formation [Jukkola et al., 1991; Melkko et al., 1990]. We were able to show that the PICP secretion correlates well with an increase in collagen accumulation in primary human bone cells [Siggelkow et al., 1998c]. These results are in accordance with the rat cell system, where a decrease in collagen accumulation relative to time in culture was seen. Comparable data were reported in the chicken osteoblast model. In their extensive investigation of the kinetics of collagen metabolism, Gerstenfeld et al. [1988] analyzed the

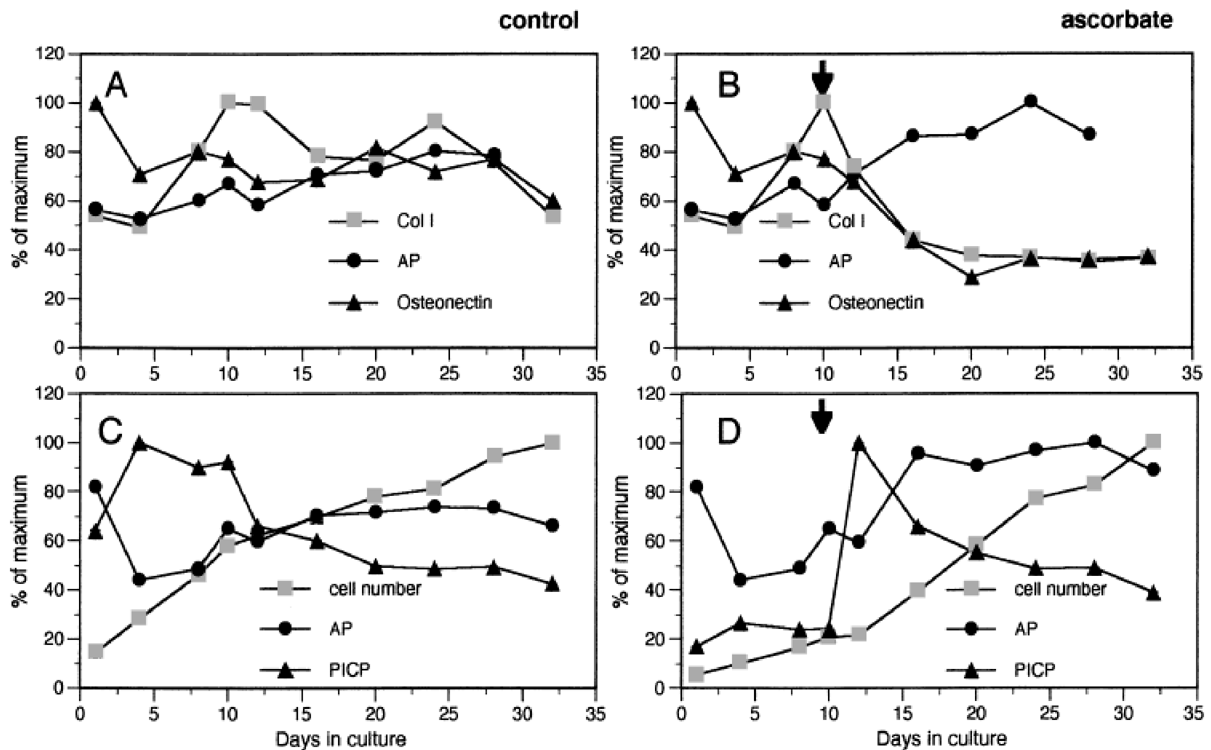

Fig. 3. Mean values, plotted relative to the maximal expression of each investigated parameter for control (left, **A,C**) or ascorbate-treated cultures **(B,D)**. In contrast with Figure 2, the mean value is now set relative to 100% instead of showing the mean ±SEM of all evaluated values. Depicted are gene expression (upper part) of procollagen I (Col I), alkaline phosphatase (AP), and osteonectin **(A,B)**. **C,D:** Values for cell number, for alkaline phosphatase activity (AP), and for procollagen I-peptide secretion (PICP).

synthesis of newly formed collagen and found a rapid decrease after confluence. In human calvarial cells, quantitative measurements of protein or mRNA-level of the characteristic osteoblastic proteins relative to time in culture were not taken [de Pollak et al., 1997].

The activity of AP decreased significantly from day 1 to 4 ($P < 0.05$) and increased to 1.5-fold by day 32 in culture, but due to high variation this change to baseline activity did not reach significance (Figs. 2C, 3C). Ascorbate stimulated AP activity after 4 days beginning at day 16 ($P < 0.05$) with no further change until day 32 (Fig. 3C). Therefore, under basal conditions, the human osteoblasts in our culture show no capability to differentiate further in culture, while ascorbate induced AP activity in these cells.

In the rat system, the maximum increase in accumulated collagen was followed by a 4-fold increase in AP activity [Owen et al., 1990]. The AP activity, which closely followed the AP mRNA expression was maximal in rat osteoblasts [Owen et al., 1990] before the onset of any mineralization. AP activity in our cell system was maximal at day 28 in culture. This is clearly in contrast to all other systems investigated so far, which show a much earlier maximal activity. The decrease in AP activity seemed to be related to proliferation in rat calvarial osteoblasts [Owen et al., 1990], cessation of proliferation even appeared to be required for the decrease in AP and the initiation of tissue-specific gene expression. In our system, no decrease and, probably because of the use of ascorbate, not even a plateauing of proliferation, was observed, possibly preventing the decrease of AP expression.

Expression of mRNA for Bone-Related Proteins AP and Procollagen I During Cell Differentiation

According to the developmental sequence suggested by Owen, the expression pattern of mRNA for osteoblast-related genes is characteristic in the various stages of differentiation [Owen et al., 1990; Stein and Lian, 1993; Stein et al., 1990]. Therefore, we investigated the gene expression for the matrix proteins procollagen I and osteonectin, which are maximally expressed during proliferation. As an indicator for matrix maturation, we used alkaline phosphatase and, for mineralization, osteocalcin gene expression. Under basal conditions, the procollagen I mRNA expression in the human primary osteoblasts started at 50% of maximum levels at day 4 to maximal levels at day 10 (Figs. 2E, 3A; $P < 0.05$). This was followed by a progressive decrease to 36% of maximum at day 32 (Figs. 2E, 3A). Ascorbate inhibited procollagen I mRNA expression with a maximum effect at days 20 and 24 (day 24, $P < 0.05$) diminishing thereafter with no effect at day 32. Under the influence of ascorbate, procollagen I mRNA decreased over 6 days to 44% at day 16, and further to the minimal value of 36% of maximum expression (day 10) at day 32 (Fig. 3B). The maximum expression of procollagen mRNA at confluence and the decrease thereafter is in accordance with results in the rat system [Owen et al., 1990]. Interestingly, in MC3T3-E1 cells maximal levels of expression of mRNA for bone matrix proteins also decreased after confluence [Choi et al., 1996].

The effect of ascorbate inducement in pHOB in both the proliferation and downregulation of procollagen mRNA is surprising. This finding supports the interpretation that the PICP peak resulted from processed preformed procollagen molecules released under the influence of ascorbate. In rat osteoblasts, the downregulation of procollagen mRNA parallels the proliferation rate [Owen et al., 1990]. The inhibiting effect of ascorbate on procollagen I mRNA beginning at day 12 in culture has not been shown up to now. To our knowledge, there are no other studies on gene expression in a human osteoblast cell system.

The mRNA levels of osteonectin decreased from early proliferation (day 1) to day 16 and remained constant thereafter until late differentiation (Figs. 2D, 3A). Under the influence of ascorbate, inhibition of osteonectin mRNA expression was significant from day 20 until day 32 ($P < 0.01$ and $P < 0.05$, Fig. 2D). Osteonectin mRNA expression decreased to 29% of maximal (day 1) levels at day 20 and remained constant thereafter (Fig. 3B). Therefore, under these conditions, the osteonectin expression parallels exactly the procollagen I expression. In rat osteoblasts and in MC3T3-E1 cells, a parallel expression sequence for collagen I, fibronectin and TGF-β was demonstrated [Choi et al., 1996; Owen et al., 1990]. We are not aware of the sequence of osteonectin expression in rat calvarial cells during in vitro differentiation, but in MC3T3-E1 cells, osteonectin mRNA expression paralleled procollagen, although both showed a different pattern of maximal expres-

sion compared with the rat or human system [Choi et al., 1996].

Under basal control conditions, the AP mRNA expression increased from day 4 to 28 1.5-fold ($P < 0.05$), demonstrating further differentiation with culture time, although at a low level (Fig. 2F). Ascorbate had a stimulating influence on AP mRNA expression, although this effect did not reach significant levels ($P = 0.074$, Fig. 2F). AP gene expression started at 53% (day 4) of maximum, while reaching the 100% expression at day 24 in culture with a decrease to 87% thereafter (Fig. 3B). The time point of maximal expression at day 24 in culture clearly occurred later than in other systems investigated. In rat calvarial cells, AP mRNA was maximally induced after the plateauing of proliferation at day 16 [Owen et al., 1990]. In our system, the proliferative period was effectively prolonged by ascorbate (Fig. 2A). If the correlation between proliferation and AP expression was valid in the human system, we should have seen a prolongation of the AP expression pattern. In fact, we can confirm the proposed correlation between AP expression and proliferation by showing an increasing AP expression after ascorbate stimulation (Fig. 3F). However, the absolute changes of AP expression are lower than described for the rat system. In the MC3T3-E1 cells, AP mRNA expression has not yet been investigated [Choi et al., 1996].

Expression of Osteocalcin in pHOB

Osteocalcin protein could not be detected above background levels in FCS treated cultures, nor could we detect osteocalcin mRNA expression by Northern blot using rehybridized Northern filter. However, low levels were detectable by PCR, indicating a low basal expression during the whole period of observation.

To investigate whether osteocalcin is expressed at all, we measured osteocalcin protein secretion and mRNA expression in pHOB cultures treated at confluence (day 10) with 1,25-dihydroxyvitamin-D_3 for 48 h (Fig. 4). In this experiment, the incubation medium did not contain any FCS, and osteocalcin protein was already detectable under control conditions with low basal levels. 1,25-dihydroxyvitamin-D_3 stimulated Oc to 13-fold levels ($P < 0.001$) above control. mRNA expression for osteocalcin was only faintly detectable in Northern blots of control cultures, but could be induced to 50-fold levels ($P < 0.001$) by 1,25-dihydroxyvitamin-D_3

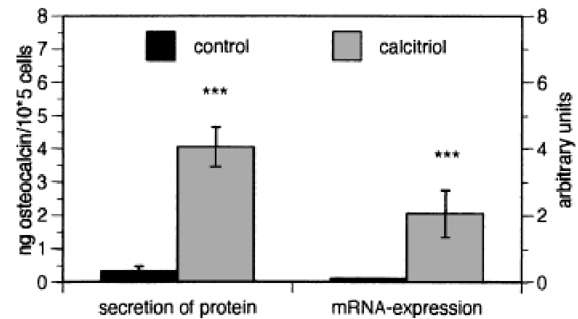

Fig. 4. Osteocalcin secretion and mRNA expression stimulated by 10^{-8} M 1,25-$(OH)_2$-D_3 for 48 h, added at confluence (day 10) compared with control. Values are mean and SEM for eight cultures. Note that, in control cultures, a basal osteocalcin secretion was measurable, whereas only faint mRNA expression could be detected. Oc protein and gene expression were highly stimulated by 1,25-$(OH)_2$-D_3 ($P < 0.001$, Wilcoxon).

at day 10 in culture demonstrating the osteoblastic nature of the cells.

To investigate whether this low level of expression of osteocalcin was attributable to the low production of few differentiated cells or a low production of all cells we determined the number of Oc-positive cells by FACS in three other primary osteoblast cell cultures grown under identical conditions. Osteocalcin is an intracellular antigen; therefore, cells had to be permeabilized before analysis. After permeabilization, we were able to demonstrate an increase in Oc-positive cells from 5.5% to 61.4% at day 6 in culture. A further increase to 96% Oc-positive cells from day 6 to 10 (confluence) suggested that the culture consisted mainly of osteoblasts producing only small amounts of osteocalcin.

In rat primary cells, osteocalcin was not detectable before induction of mineralization, and its expression depended on mineral accumulation which seems to be essential to induce the mineralization phase. When mineral accumulation was retarded because of the lack of bGP, an increase in Oc was not detectable before day 25 in rat osteoblasts. We did not find a significant increase in Oc expression or protein secretion in pHOB cells cultured without bGP even after day 25.

Expression of Lipoprotein Lipase

Over the last few years, increasing information has been made available concerning the differentiation potential of osteoblast progenitors [Cheng et al., 1994; Kassem et al., 1994; Oreffo et al., 1997; Rickard et al., 1996]. Multi-

potent stromal fibroblastic stem cells in the bone marrow can differentiate into fibroblastic, osteogenic, adipogenic, and reticular cells. Recently, Nutall et al. [1998] succeeded in showing that cells cultured from human trabecular bone are not only osteogenic but are also able to undergo adipocyte differentiation under defined culture conditions. We tested our osteoblast-like cells for the expression of lipoprotein lipase, a marker of the adipocytic phenotype. There was no significant expression of lipoprotein lipase at day 10 and 32 in cultures of the nine patients as measured by RT-PCR. Therefore, the low osteocalcin expression at day 32 in culture is not caused by a dedifferentiation of osteoblasts to preadipocytes.

DISCUSSION

Primary human osteoblast cultures from healthy young male donors, established with a highly standardized explant technique, show an expression pattern of bone-related proteins during proliferation and matrix formation somewhat similar to the developmental sequence in rat osteoblasts proposed by Stein and Lian's group [Owen et al., 1990; Stein and Lian, 1993; Stein et al., 1990]. Because of the missing mineralization and nodule formation in our system, the last stage of the sequence was not expressed. In contrast to the similarities in expression pattern for bone-related proteins in the rat and human system, the regulation of proliferation seems to be different in both systems. In the following discussion, we first try to assign the developmental stages defined by Owen and colleagues to our data by using the gene expression profile only. In the second part, we discuss the contrasting proliferation data and the consequences for the definition of a developmental sequence.

In rat calvarial cells, the "proliferation stage" is characterized by active proliferation and the maximal expression of genes associated with the formation of the extracellular matrix, such as collagen I. This period was related up to day 12 in culture [Owen et al., 1990]. At this day, procollagen I is 80% and AP expression 50% of maximum and proliferation 2 days before plateauing. In the pHOB system, we found a similar procollagen I/AP constellation around day 12; however, there was no plateauing of proliferation throughout the experiment (Fig. 5A). In pHOB, procollagen I and osteonectin mRNA correlated closely with the rat system, expressed to a similar level at day 12 in culture, when AP was 50% of maximum. Using only the given expression of collagen I and AP for the definition of the "proliferation" and "maturation stage" proposed by Owen and colleagues, the end of the "proliferation stage" in the human system could possibly be designated at day 12 (Fig. 5A).

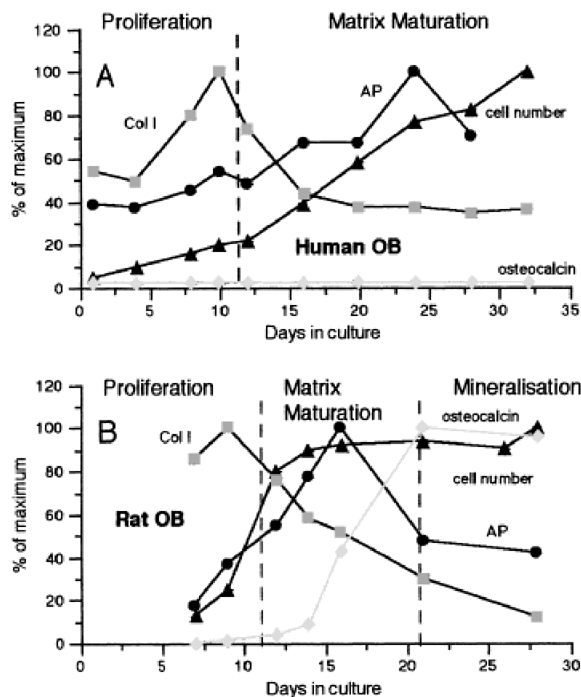

Fig. 5. Comparison of the developmental sequence defined by the expression of alkaline phosphatase (AP), procollagen I (Col I), and osteocalcin gene expression in ascorbate-treated cultures in relation to proliferation. The stages in the primary human osteoblast (**A:** human OB) were defined by the model proposed by Owen for rat primary osteoblasts (**B:** rat OB). The human culture only reaches the middle of the "matrix maturation phase," compared with the rat system.

The first part of the next phase was associated with the formation of extracellular matrix in rat calvarial cells and was characterized by an increase in the expression of alkaline phosphatase paralleled by its enzyme activity. During that phase AP expression became maximal, whereas osteocalcin was not expressed significantly. This phase was limited from day 12 to16 in culture (Fig. 5B). In pHOB AP expression and activity was maximal at day 24 while, in accordance with the prerequisite of this phase, the osteocalcin expression was low. Therefore, the first part of the "matrix formation phase" in pHOB, as defined by the gene expression profile, would be assigned to the phase between days 12 and 24 (Fig. 5A). The end of the "matrix

maturation stage" in rat osteoblasts is characterized by a maximal increase in osteocalcin expression which indicates the beginning of the "mineralization stage" at day 22. Without the addition of bGP, the increase in OC expression is prolonged to day 25 [Owen et al., 1990]. Although we investigated our cells until day 32 in culture no change in Oc expression was detectable. Therefore, up to day 32, we did not find an index for the transition into the mineralization phase in our culture. If only the sequence of mRNA expression of AP, procollagen I, osteonectin, and osteocalcin is used to define the different phases, we would assume that, in the pHOB system, only the "proliferation" and the first part of the "matrix maturation phase" are definable, because no increase in osteocalcin expression was detectable (Fig. 5).

In addition to the species differences, some methodological variations must be discussed in further detail. In rat calvarial cells, the lack of bGP caused a 10-day elongated expression sequence, whereas no influence on proliferation and AP gene expression was detected [Owen et al., 1990]. In addition to a deficiency of bGP in our cultures as compared with the rat calvarial cell system, we used cells in the second passage and plated them at 20% lower density. This difference in experimental conditions was necessary because of the low cell yield of primary human osteoblasts. As shown by Owen et al. [1990], subcultivated cells remain competent to express the developmental sequence, but a lengthening of the time course was observed. Furthermore, with the lower plating density, our cells reached confluence at day 10 compared with day 7 in the rat system. The necessary changes in our experimental setup most probably explain the demonstrated shift in matrix maturation in pHOB. The attenuated absolute increase in AP expression might be due to the use of second-passage cells. In addition to these variables, the different localization of the bone specimens could in itself cause differences in the expresssion sequence. Our bone explants were taken from the iliac crest, while the rat osteoblasts were established from calvaria. Therefore, our results might be specific for osteoblasts derived from the iliac crest. Differences in the composition and behavior of osteoblasts from different sources were shown before [Kasperk et al., 1995].

In contrast to the corresponding expression of bone-related proteins in rat calvarial cells and primary human osteoblasts, the kinetic of proliferation is different in each system. In the rat system, the maximum expression of proliferation-related genes preceded the plateauing of cell proliferation by 4 days. Ascorbate inhibited cell proliferation in rat calvarial cells, as shown by accentuated plateauing with a lower cell number [Owen et al., 1990]. By contrast, ascorbate stimulated cell proliferation in pHOB cells up to 4-fold, as demonstrated by cell number and total DNA measurements. There was no final plateauing during the investigated culture time in ascorbate-treated human bone cell cultures. In the rat system, the decrease in proliferation was a prerequisite for the progress to the after developmental stage. In pHOB the expression profile proceeded to the matrix maturation stage independently from this prolonged proliferation. This points to a regulatory difference between rat and human osteoblasts concerning the progress from the proliferative to the matrix maturation phase and questions the use of this developmental sequence in the human system. Therefore, the corresponding stages might be termed differently for the human system, e.g., "early and late proliferation phase."

In conclusion, our results showed a time-dependent expression of AP and procollagen I mRNA similar to the rat system, although the quantitative changes of expression were attenuated. The assignment of the transition from "proliferation" to "matrix maturation stage" as defined for the rat system was only possible if based on gene expression, because proliferation was not plateauing during the investigated culture time. Mineralization was not observed, as shown by the missing increase in Oc expression.

ACKNOWLEDGMENTS

The authors are grateful to the Department of Surgery for providing the bone samples. We also thank Phillip Kurlbaum for help with the manuscript.

REFERENCES

Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF. 1991. Complementary DNA sequencing: expressed sequence tags and human genome project. Science 252: 1651–1656.

Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS. 1990. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol 143:213–221.

Ashton BA, Abdullah F, Cave J, Williamson M, Sykes BC, Couch M, Poser JW. 1985. Characterization of cells with high alkaline phosphatase activity derived from human bone and marrow: preliminary assessment of their osteogenicity. Bone 6:313–319.

Auf'm Kolk B, Hauschka PV, Schwartz ER. 1985. Characterization of human bone cells in culture. Calcif Tissue Int 37:228–235.

Barbu V, Dautry F. 1989. Northern blot normalization with a 28S rRNA oligonucleotide probe. Nucleic Acids Res 17:7115–7125.

Battmann A, Battmann A, Jundt G, Schulz A. 1997. Endosteal humn bone cells (EBC) show age-related activity in vitro. Exp Clin Endocrinol Diabetes 105:98–102.

Bellows CG, Aubin JE, Heersche NM, Antosz ME. 1986. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154.

Beresford JN, Gallagher JA, Poser JW, Russell RGG. 1984. Production of osteocalcin by human bone cells in vitro. Effects of 1,25-$(OH)_2D_3$,24,25-$(OH)_2D_3$, parathyroid hormone, and glucocorticoids. Metab Bone Dis Rel Res 5:229–234.

Boyan BD, Schwartz Z, Bonewald LF, Swain LD. 1989. Localization of 1,25-(OH)2D3-responsive alkaline phosphatase in osteoblast-like cells (ROS 17/2.8, MG 63, and MC3T3) and growth cartilage cells in culture. J Biol Chem 264:11879–11886.

Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wozney JM. 1986. Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. EMBO J 5:1885–1890.

Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. 1994. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, Jo JS, Ryoo HM. 1996. Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. J Cell Biochem 61:609–618.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA-isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Choong PF, Martin TJ, Ng KW. 1993. Effects of ascorbic acid, calcitriol, and retinoic acid on the differentiation of preosteoblasts. J Orthop Res 11:638–647.

Collin P, Nefussi JR, Wetterwald A, Nicolas V, Boy-Lefevre M-L, Fleisch H, Forest N. 1992. Expression of collagen, osteocalcin, and bone alkaline phosphatase in a mineralizing rat osteoblastic cell culture. Calcif Tissue Int 50:175–183.

de Pollak C, Arnaud E, Renier D, Marie PJ. 1997. Age-related changes in bone formation, osteoblastic cell proliferation, and differentiation during postnatal osteogenesis in human calvaria. J Cell Biochem 64:128–39.

Dean DD, Schwartz Z, Bonewald L, Muniz OE, Morales S, Gomez R, Brooks BP, Qiao M, Howell DS, Boyan BD. 1994. Matrix vesicles produced by osteoblast-like cells in culture become significantly enriched in proteoglycan-degrading metalloproteinases after addition of beta-glycerophosphate and ascorbic acid. Calcif Tissue Int 54:399–408.

Dodds RA, Merry K, Littlewood A, Gowen M. 1994. Expression of mRNA for IL1β, IL6 and TGFβ1 in developing human bone and cartilage. J Histochem Cytochem 42: 733–744.

Evans CE, Galasko CSB, Ward C. 1990. Effect of donor age on the growth in vitro of cells obtained from human trabecular bone. J Orthop Res 8:234–237.

Feinberg AP, Vogelstein B. 1983. A technique for radiolabeling DNA restriction fragments to a high specific activity. Anal Biochem 132:180–182.

Franceschi RT, Iyer BS, Cui Y. 1994. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. J Bone Miner Res 9:843–854.

Gehron Robey P, Termine JD. 1985. Human bone cells in vitro. Calcif Tissue Int 37:453–460.

Gerstenfeld LC, Chipman SD, Glowacki J, Lian JB. 1987. Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev Biol 122:49–60.

Gerstenfeld LC, Chipman SD, Kelly CM, Hodgnes KJ, Lee DD, Landis WJ. 1988. Collagen expression, ultrastructural assembly and mineralization in cultures of chicken embryo osteoblasts. J Cell Biol 106:979–989.

Gotoh Y, Fujisawa K, Satomura K, Nagayama M. 1995. Osteogenesis by human osteoblastic cell in diffusion chamber in vivo. Calcif Tissue Int 56:246–251.

Gundle R, Beresford JN. 1995. The isolation and culture of cells from explants of human trabecular bone. Calcif Tissue Int 56:S8–S10.

Harada S, Matsumoto T, Ogata E. 1991. Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. J Bone Miner Res 6:903–908.

Jukkola A, Risteli J, Risteli L. 1991. Effect of dextran on synthesis, secretion and deposition of type III procollagen in cultured human fibroblasts. Biochem J 279:49–54.

Jung T, Schauer U, Heusser C, Neumann C, Rieger C. 1993. Detection of intracellular cytokines by flow cytometry. J Immunol Methods 159:197–207.

Kassem M, Risteli L, Mosekilde L, Melsen F, Eriksen EF. 1991. Formation of osteoblast-like cells from human mononuclear bone marrow cultures. Apmis 99:269–274.

Kassem M, Brixen K, Mosekilde L, Eriksen EF. 1994. Human marrow stromal osteoblast-like cells do not show reduced responsiveness to in vitro stimulation with growth hormone in patients with postmenopausal osteoporosis. Calcif Tissue Int 54:1–6.

Kasperk C, Wergedal J, Strong D, Farley J, Wangerin K, Gropp H, Ziegler R, Baylink DJ. 1995. Human bone cell phenotypes differ depending on their skeletal site of origin. J Clin Endocrinol Metab 80:2511–2517.

Melkko J, Niemi S, Risteli L, Risteli J. 1990. Radioimmunoassay of the carboxyterminal propeptide of human type I procollagen. Clin Chem 36:1328–1332.

Mundlos S. 1994. Expression patterns of matrix genes during human skeletal development. Prog Histochem Cytochem 28:1–47.

Nefussi J-R, Boy-Lefevre ML, Boulekbach H, Forest N. 1985. Mineralization in vitro of matrix formed by osteoblaste isolated by collagenase digestion. Differentiation 29:160–168.

Nefussi JR, Brami G, Modrowski D, Oboeuf M, Forest N. 1997. Sequential expression of bone matrix proteins during rat calvaria osteoblast differentiation and bone nodule formation in vitro. J Histochem Cytochem 45:493–503.

Nomura S, Wills AJ, Edwards DR, Heath JK, B.L.M. H. 1988. Developmental expression of 2ar (osteopontin) and SPARC(osteonectin)RNA as revealed by in situ hybridization. J Cell Biol 106:441–450.

Nutall ME, Patton AJ, Olivera DL, Nadeau DP, Gowen M. 1998. Human trabecular bone cells are able to express both osteoblasic and adipocytic phenotype: implications for osteopenic disorders. J Bone Miner Res 13:371–382.

Oreffo RO, Virdi AS, Triffitt JT. 1997. Modulation of osteogenesis and adipogenesis by human serum in human bone marrow cultures. Eur J Cell Biol 74:251–261.

Owen M. 1985. Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA, editor. Bone and mineral research annual. Amsterdam: Exerpta Medica. p 1–25.

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS. 1990. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430.

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB. 1991. Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology 128:1496–1504.

Pockwinse SM, Stein JL, Lian JB, Stein GS. 1995. Developmental stage-specific cellular responses to vitamin D and glucocorticoids during differentiation of the osteoblast phenotype: interrelationship of morphology and gene expression by in situ hybridization. Exp Cell Res 216:244–260.

Quarles DL, Yohay DA, Lever LW, Caton R, Wenstrup RJ. 1992. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 7:683–692.

Rago R, Mitchen J, Wildling G. 1990. DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. Anal Biochem 191:31–34.

Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL. 1996. Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res 11:312–324.

Robinson JA, Harris SA, Riggs BL, Spelsberg TC. 1997. Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919–2927.

Sautier JM, Nefussi JR, Forest N. 1993. In vitro differentiation and mineralization of cartilaginous nodules from enzymatically released rat nasal cartilage cells. Biol Cell 78:181–189.

Schulz R, Bethäuser H, Stempka L, Heilig b, Moll A, Hüfner M. 1989. Evidence for immunological differences between circulating and thyroid tissue-derived thyroglobulin in men. Eur J Clin Invest 19:459–463.

Siggelkow H, Benzler K, Atkinson MJ, Hüfner M. 1998a. Phenotypic stability of primary human osteoblast-like cells at different cell densities and passage numbers. Exp Clin Endocrinol Diabetes 106:217–225.

Siggelkow H, Hilmes D, Rebenstorff K, Kurre W, Hüfner M. 1998b. Analysis of human primary bone cells by fluorescence activated cell scanning: methodological problems and preliminary results. Clin Chim Acta 272:111–125.

Siggelkow H, Neubauer K, Schulz H, Krüger M, Ramadori G, Hüfner M. 1998c. Einfluß von Ascorbat auf das Kollagenprozessing und die Sekretion von Procollagen-I-Peptid in primären humanen Osteoblasten. Osteologie 7:31.

Siggelkow H, Niedhart C, Kurre W, Ihbe A, Schulz A, Atkinson M, Hüfner M. 1998d. In vitro differentiation potential of a new human osteosarcoma cell line HOS 58. Differentiation 63:81–91.

Siggelkow H, Schulz H, Hüfner M. 1995. Investigation on the variability of human osteoblast-like cultures. Calcif Tissue Int 56:438.

Spindler KP, Shapiro DB, Gross SB, Brighton CT, Clark CC. 1989. The effect of ascorbic acid on the metabolism of rat calvarial bone cells in vitro. J Orthop Res 7:696–701.

Stein GS, Lian JB. 1993. Molecular mechanisms mediating developmental and hormone-regulated expression of genes in osteoblasts: an integrated relationship of cell growth and differentiation. In: Noda M, editor. Cellular and molecular biology of bone. Tokyo: Academic Press. p 47–95.

Stein GS, Lian JB, Owen TA. 1990. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4:3111–3123.

Sugimoto T, Nakada M, Fukase M, Imai Y, Kinoshita Y, Fujita T. 1986. Effects of ascorbic acid on alkaline phosphatase activity and hormone responsiveness in the osteoblastic osteosarcoma cell line UMR-106. Calcif Tissue Int 39:171–174.

Tenenbaum HC, Heersche JNM. 1982. Differentiation of osteoblasts and formation of mineralized bone in vitro. Calcif Tissue Int 34:76–79.

Vuorio T, Mäkelä TJ, Kähäri V-M, Vuorio E. 1987. Coordinated regulation of type I and type III collagen production and mRNA levels of pro $\alpha1$(I) and pro $\alpha2$(I) collagen in cultured morphea fibroblasts. Arch Dermatol Res 279: 154–160.

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

Weiss MJ, Henthorn PS, Lafferty MA, Slaughter C, Raducha M, Harris H. 1986. Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. Proc Natl Acad Sci USA 83:7182–7186.

Yoon K, Buenaga R, Rodan G. 1987. Tissue specificity and developmental expression of rat osteopontin. Biochem Biophys Res Commun 148:1129–1136.