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Fetal bone cells for tissue engineering

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Abstract

We envision the use of human fetal bone cells for engineered regeneration of adult skeletal tissue. A description of their cellular function is then necessary. To our knowledge, there is no description of human primary fetal bone cells treated with differentiation factors. The characterization of fetal bone cells is particularly important as the pattern of secreted proteins from osteoblasts has been shown to change during aging. In the first part of this work, human primary fetal bone cells were compared to adult bone cells and mesenchymal stem cells for their ability to proliferate and to differentiate into osteoblasts in vitro. Cell proliferation, gene expression of bone markers, alkaline phosphatase (ALP) activity, and mineralization were analyzed during a time-course study. In the second part of this paper, bone fetal cells behavior exposed to osteogenic factors is further detailed. The doubling time of fetal bone cells was comparable to mesenchymal stem cells but significantly shorter than for adult bone cells. Gene expression of cbfa-1, ALP, α1 chain of type I collagen, and osteocalcin were upregulated in fetal bone cells after 12 days of treatment, with higher inductions than for adult and mesenchymal stem cells. The increase of ALP enzymatic activity was stronger for fetal than for adult bone cells reaching a maximum at day 10, but lower than for mesenchymal stem cells. Importantly, the mineralization process of bone fetal cells started earlier than adult bone and mesenchymal stem cells. Proliferation of fetal and adult bone cells was increased by dexamethasone, whereas 1α,25-dihydroxyvitamin D_3 did not show any proliferative effect. Mineralization studies clearly demonstrated the presence of calcium deposits in the extracellular matrix of fetal bone cells. Nodule formation and calcification were strongly increased by the differentiation treatment, especially by dexamethasone. This study shows for the first time that human primary fetal bone cells could be of great interest for bone research, due to their fast growth rate and their ability to differentiate into mature osteoblasts. They represent an interesting and promising potential for therapeutic use in bone tissue engineering.
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Keywords: Bone tissue engineering; Differentiation; Human fetal cells; Mesenchymal stem cells; Osteoblast

Introduction

To address the need of an increasing number of patients who require bone for skeletal reconstruction, surgeons can overcome the disadvantages linked to auto- or allo-bone grafts by choosing the tissue engineering approach [1,2].

This strategy consists of three necessary elements: (1) cells with a high osteogenic potential, easy to obtain and to handle under standardized conditions, (2) osteogenic growth factors, and (3) a synthetic three-dimensional scaffold which gives the construct sufficient mechanical properties for loading and facilitates vascularization.

The cell origin and type are essential aspects in bone tissue engineering. This has been recapitulated by different articles, particularly in the field concerning bone marrow-

Abbreviations: 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; ALP, alkaline phosphatase; cbfa-1, core binding factor alpha 1; MSC, mesenchymal stem cells.

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derived stem cells [3–5]. As one of every 100,000 nucleated cells derived from bone marrow is a stem cell, a procedure of isolation is required to decrease the volume of material injected [6]. Recently, a highly purified population of stromal stem cells derived from human bone marrow was described [7]. Exposure to growth factors or the combination with fibrillar collagen increased the proliferation of stem cells and their differentiation into osteoblastic cells [8,9]. However, the number of human mesenchymal stem cells with osteogenic potential was shown to decrease early during aging [10], and the proliferation of human bone marrow stromal cells was reported to be negatively correlated with the age of the donor in vitro [11].

Periosteal cells have been widely used in bone tissue engineering by isolating osteoprogenitor cells from the patient's periosteum and seeding them into bioresorbable scaffolds [12–16].

Fetal-associated tissues such as placenta, amniotic liquid, or umbilical cord are described to be potential source of cells for tissue engineering [17–20]. In contrast to embryonic derived up to the end of the eighth week, beginning at the ninth week, cells are considered as fetal and tissue dissection as organ donation. Human fetal liver cells have been already used for transplantation to treat severe immunodeficiencies, hematological disorders, and inborn errors of metabolism, when there was no perfectly matched donor for marrow transplantation [21]. Neuronal affections such as Huntington [22] or Parkinson disease [23] have been treated by transplantation of fresh fetal neuroblasts. Unfortunately, these cells are difficult to expand in culture and have to be transplanted freshly therefore needing large quantities of fresh tissue [24]. Recently, human fetal skin cells derived from one cell bank (1–4 cm^2 tissue results in over 10.5 million fetal skin constructs) were used in clinical trials, and new advances in tissue therapy are possible with cellular constructs obtained from ex vivo cultures (Hohlfeld, J. submitted). Engineered regeneration of human skeletal adult tissues could be also developed using human fetal bone cells. To evaluate their potential integration in a bone engineering strategy, a biological characterization of these cells is necessary. To our knowledge, there is no available description of human primary fetal bone cells behavior when treated with factors of differentiation.

Age-dependent biology of osteoblasts is generally accepted [25]. The particular biology of human primary fetal bone cells was partially demonstrated with cells isolated from calvaria [26]. They were found to secrete primarily matrix constituents proteins in culture, whereas adult cells produce additionally proteins involved in matrix turnover. Age-dependent differences regarding the osteoblastic synthesis of osteoanabolic peptides and their impact on the regeneration of osseous defects were observed with a rat calvaria model [27].

Osteoblast development follows three main phases: proliferation, matrix synthesis, and mineralization [28]. Factors for differentiation of osteoblasts are well described [29,30] and include ascorbic acid, β-glycerophosphate, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25\text{-}(OH)_2D_3$), and dexamethasone.

The aim of this work was to study the characteristics of human primary fetal bone cells for a better comprehension of their biology in vitro and to evaluate their potential use for tissue engineering in comparison to adult bone cells and mesenchymal stem cells. In the first part of this paper, fetal bone cells, adult bone cells, and mesenchymal stem cells are compared for their self-renewal capacity and their osteogenic induction when treated with differentiation mix. Their osteoblastic phenotype is presented at the gene expression level, such as the core binding factor alpha 1 (cbfa-1), a critical transcription factor for osteoblast differentiation and function [31–33]; alkaline phosphatase (ALP) [34]; α1 chain of type I collagen, which represents 85–90% of the total organic bone matrix [35]; and osteocalcin, which constitutes 1–2% of the total protein of bone [36]. At the protein level, ALP enzymatic activity and the ability of matrix mineralization are observed. In the second part of this paper, the effects of different osteoinductor factors on human primary fetal bone cells are examined to further characterize their ability to differentiate.

Materials and methods

Cell sources

Human fetal and adult bone cells were obtained from our bank of bone cells comprising 32 adult donors (18 females and 14 males) and 4 fetal donors (1 female and 3 males) at the end of March 2004. More than 250 primary total hip replacements with potentially interesting orthopedic tissue samples were performed during the year 2002 at the Hôpital Orthopédique de la Suisse Romande. Documents relative to the patients were consulted to assure no major disease, medications, or alcohol consumption. Bone samples were extensively collected, and care was taken to obtain healthy bone samples. In particular, the selected adult bone cells are coming from donors without bone necrosis. In this study, human trabecular bone biopsies at femoral locations obtained from 31-, 50-, and 60-year-old adult women subsequent to orthopedic surgery and from fetus of 13, 14, and 16 weeks' gestational age following voluntary interruption of pregnancy were used. Biopsies were obtained in accordance with the Ethics Committee of University Hospital in Lausanne (Ethical Protocol 51/01). Primary osteoblast cultures were established by rinsing the tissue first with PBS (containing penicillin and streptomycin). Afterwards, bone samples were mechanically dissociated with a scalpel blade and transferred to 10-cm culture grade plates where cell outgrowth was seen within 2 to 5 days under normal culture conditions : DMEM Glutamax (Invitrogen, Carlsbad, CA), 10% FCS (Sigma–Aldrich, St. Louis, MO). Commercially available mesenchymal stem

cells (Cambrex Bio Science, Walkersville, MD), isolated from the posterior iliac crest of the pelvic bone from young and healthy volunteers, were tested parallelly.

Cell culture

Fetal and adult bone cells were maintained in culture by passaging once a week and culturing at 37°C in an humidified, 5% CO_2 atmosphere in DMEM Glutamax, 10% FCS. Mesenchymal stem cells were expanded in MSCGM growth medium (Cambrex Bio Science). Culture media were changed twice a week.

For the comparison between fetal bone cells, adult bone cells, and mesenchymal stem cells, 1α,25-$(OH)_2D_3$ 10 nM (Alexis Biochemicals, San Diego, CA), dexamethasone 10 nM (Sigma–Aldrich), additionally to L-ascorbic acid 50 μg/ml (Sigma–Aldrich), and β-glycerophosphate 1 mM (Sigma–Aldrich) were added in their respective growth medium to obtain the full differentiation mix. For the characterization of fetal bone cells, osteogenic factors were tested as follows: ascorbic acid 50 μg/ml and β-glycerophosphate 1 mM (minimal differentiation mix); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and dexamethasone 10 nM (dexamethasone mix); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and 1α,25-$(OH)_2D_3$ 10 nM (vitamin D_3 mix); and ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). In general, passage 3 was used for all experiments. Concerning fetal bone cells, the same assays were performed at passage 6 to verify their multilineage capability. Each procedure was repeated three times, starting from a new vial of cells which were stored in liquid N_2. Unless otherwise stated, results presented were obtained with the 16-week-old donor for the fetal source and the 50-year-old donor for the adult bone cells (Table 1). Similar data were obtained with two other fetal and adult donors.

Table 1
Comparison of the doubling time of human fetal bone, adult bone, and mesenchymal stem cells

A. Fetal bone cells

Donor	Age (weeks)	Passage 3 (h)	Passage 6 (h)
1	16	27.0 (±0.2)	30.2 (±1.5)
2	14	22.6 (±0.5)	25.1 (±0.7)
3	13	30.3 (±0.1)	31.4 (±0.1)

B. Adult bone cells

Donor	Age (years)	Passage 3 (h)
1	60	248.7 (±20.1)
2	50	170.5 (±11.8)
3	31	151.6 (±5.6)

C. Mesenchymal stem cells

Donor	Age (years)	Passage 3 (h)
1	26	26.5 (±0.9)

Experiments were performed in triplicates and presented as mean values (±SEM).

Cell proliferation

To determine their doubling time, cells were seeded at a density of 440,000 cells/T75 flask. Every day, up to 10 days, triplicates were washed twice with PBS, and cells were detached using trypsin–EDTA (Invitrogen). Subsequently, the cell number was determined in a counting chamber (Marienfeld GmbH & Co. KG, Lauda-Koenigshofen, Germany). The growth constant and the generation time were determined during the exponential cell growth [37].

To evaluate the effects of osteogenic factors on proliferation, cells were seeded at 5000 cells/well in 96-well plates and incubated for 24 h at 37°C in a humidified, 5% CO_2 atmosphere in normal culture medium. The medium was then replaced by 100 μl/well fresh medium containing the factors of differentiation. Every second day, up to 12 days, a plate was analyzed for cell proliferation, and the medium of the remaining plates was renewed. Measurements were performed in triplicate for each time point. The relative number of viable cells in each well was determined with the CellTiter 96^R AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. This colorimetric method is based on the bioreduction of the MTS tetrazolium compound into a colored formazan product which is soluble in culture medium. It was determined to produce a linear relationship between the number of viable cells and the absorbance at 490 nm.

Briefly, 20 μl of the reagent solution was added to each well, including three wells containing only medium for background subtraction. After incubation for 90 min at 37°C, the absorbance was recorded at 490 nm using a Wallac 1420 VICTOR^2 multilabel plate reader (Wallac Oy, Turku, Finland).

RNA isolation and purification

Cells were seeded in 6-cm Petri dishes at a density of 300,000 cells/dish. Treatment began 24 h later, with culture media changed every second day. At the same time points, samples were removed for gene expression analysis. RNA isolation and purification procedures were performed using Nucleospin^R RNA II columns (Macherey-Nagel, Düren, Germany) with the furnished protocol. RNA was eluted in 40 μl of RNase-free water and stored at −80°C until required.

First strand synthesis

For each sample, 5 μl of total RNA was reverse-transcribed using the Taqman Universal polymerase chain reaction (PCR) reagents with random hexamers (Applied Biosystems, Foster City, CA). Reaction volumes were fixed

at 50 μl according to the protocol provided by the supplier. The thermal cycler PCT-0100 (MJ Research, Waltham, MA) was programmed as follows: 25°C 10 min, 48°C 30 min, and 95°C 5 min.

Real time polymerase chain reaction

Specific primers for cbfa-1, α1 chain of type I collagen, osteocalcin, ALP, and for the housekeeping 18S RNA were designed with the Primer Express® software (Applied Biosystems) and purchased from Integrated DNA Technologies (Coralville, IA). An additional sequence of 18 bp was added to the 5' end of every forward primer to use the Amplifluor® Uniprimer® technology (Intergen Discovery Products, Purchase, NY). PCR reactions were performed in 25 μl: 5 μl of first strand, 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), and 7.5 μl of the primers working solution. Thermal cycle conditions were 50°C 2 min, 95°C 10 min, then 50 cycles at 95°C 15 s, 60°C 1 min. Amplifications were monitored with the ABI Prism 7700 (Applied Biosystems). Measurements were performed in duplicates for each time point. Relative gene expressions were analyzed with the $2^{-\Delta\Delta CT}$ method [38]. Normalized to 18S RNA, each gene expression was compared with the ΔCT calibrator value from the untreated group. PCR products were purified using Wizard® SV gel and PCR Clean-Up system (Promega) and sequenced with the ABI Prism 3100 Genetic Analyser, using the Big Dye Terminator v3.1 Cycle Sequencing Kit Chemistry (Applied Biosystems). Results were in accordance with the expected sequences (data not shown).

Alkaline phosphatase activity

Cells were seeded at 5000 cells/well in 96-well plates and incubated for 24 h at 37°C in a humidified, 5% CO_2 atmosphere in normal culture medium. The medium was then replaced with 100 μl/well fresh medium containing the osteogenic factors. Every second day, up to 12 days, a plate was analyzed for the alkaline phosphatase activity, and the medium in the remaining plates was renewed at this occasion. Measurements were performed in triplicates for each time point. Alkaline phosphatase activity was determined using *p*-nitrophenol tablets as substrate (Sigma–Aldrich). The ALP activity values were normalized to the total protein content determined using the total cellular protein assay (see below). Calculations were done according to the manufacturer's instructions (Sigma, procedure n°245).

Total cellular protein

Cells were seeded at the same density as for the alkaline phosphatase activity assay, and same treatments were applied. At each time point, cells were rinsed twice with PBS, and the total protein was determined in cell lysates using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Measurements were performed in triplicates for each time point. Total protein values were quantified using a standard curve obtained with a serial dilutions of bovine serum albumin contained in the Bio-Rad Protein Assay Standard II (Bio-Rad Laboratories).

In vitro mineralization

Cells were seeded at 5000 cells/well in 96-well plates and incubated for 24 h at 37°C in a humidified, 5% CO_2 atmosphere in normal culture medium. The medium was then replaced by the differentiation factors. The experiment was performed twice, with 5 wells for each condition. The medium was renewed every second day. Up to 6 weeks, every week, a plate was analyzed for mineralization. MC3T3 E1 cells were used as positive control. The degree of in vitro calcium deposition was determined using Von Kossa staining. Briefly, the medium was removed from the wells, and the cells were rinsed three times with PBS. After washing, the

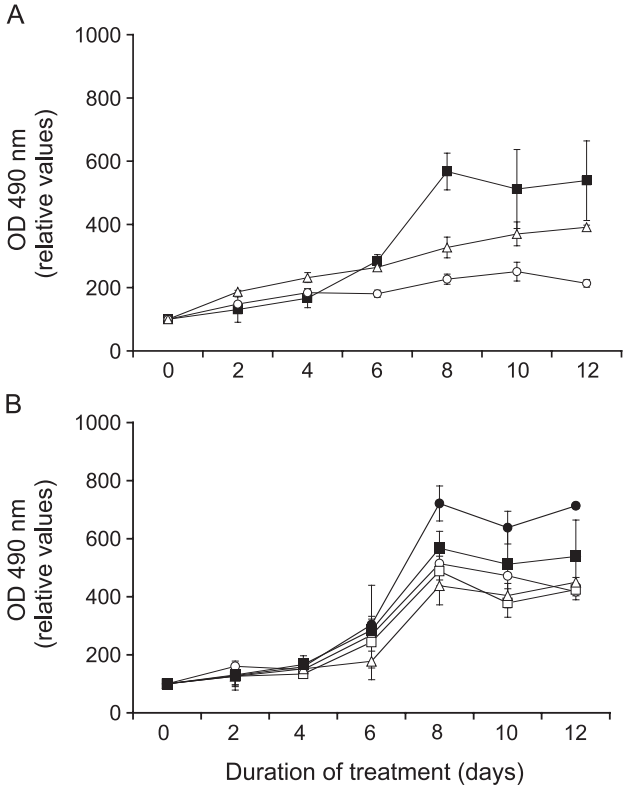

Fig. 1. (A) Proliferation curves of fetal bone cells (■), adult bone cells (○), and mesenchymal stem cells (△). Cells were treated with ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). (B) Proliferation of fetal bone treated with ascorbic acid 50 μg/ml and β-glycerophosphate 1 mM (minimal differentiation mix, □); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM and dexamethasone 10 nM (dexamethasone mix, ●); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and 1α,25-$(OH)_2D_3$ 10 nM (vitamin D_3 mix, △); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix, ■). Optical densities are expressed as ratios relative to day 0 levels. Results are shown as the mean ± SEM of three experiments performed in triplicate. Media were renewed every second day.

cells were fixed for 5 min in neutral formalin 10%. The formalin was removed, and the cells were washed three times with deionized water. The cells were then stained with $AgNO_3$ solution 5% (in water). The plates were exposed for 1 h under UV light and washed three times with water. The water was removed, and a solution of sodium-thiosulfate 5% (in water) was added for 2 min. Finally, cells were washed three times with water, and samples were examined for black clusters by light microscopy.

Scanning electron microscopy

Fetal bone cells were seeded at 100,000 cells/well in 6-well plates and treated during 6 weeks under the same conditions as described for the mineralization assay. Subsequently, cells were fixed with glutaraldehyde, followed by a dehydration procedure in ethanol. The sample surfaces were gold-coated during 2 min under 1 kV and observed using a scanning electron microscope Philips XL30 (FEI, Hillsboro, OR) at a voltage of 5 kV.

Statistical analyses

Data were analyzed using the two-way ANOVA to determine the difference between cells behaviors (XLSTAT, Addinsoft, Brooklyn, NY). The Fisher LSD test was used to determine statistical significance between sample sets. A value of $P < 0.05$ was taken as a significant difference. The same approach was used to evaluate the effects of differentiation factors on bone fetal cells. Results are shown as the mean ± SEM. Comparisons of proliferation and ALP enzymatic activity between cells are expressed as ratios of respective inductions. Effects of the different osteogenic treatments on bone fetal cells are expressed as percent (%) change versus the reference.

Results

Cell proliferation

Growth curves obtained for human primary fetal and adult bone cells show that the former proliferated considerably more rapidly than mature cells which displayed a much slower and clearly age-dependent doubling time. For fetal bone cells, it was determined by cell counting to vary between 23 and 27 h, depending on the donor (Table 1). A similar value was obtained with mesenchymal stem cells. For adult bone cells derived from a 31- and 50-year-old donor, it was determined to be 152 and 170 h, respectively, while cells coming from a 60-year-old donor showed a doubling time even as slow as 249 h.

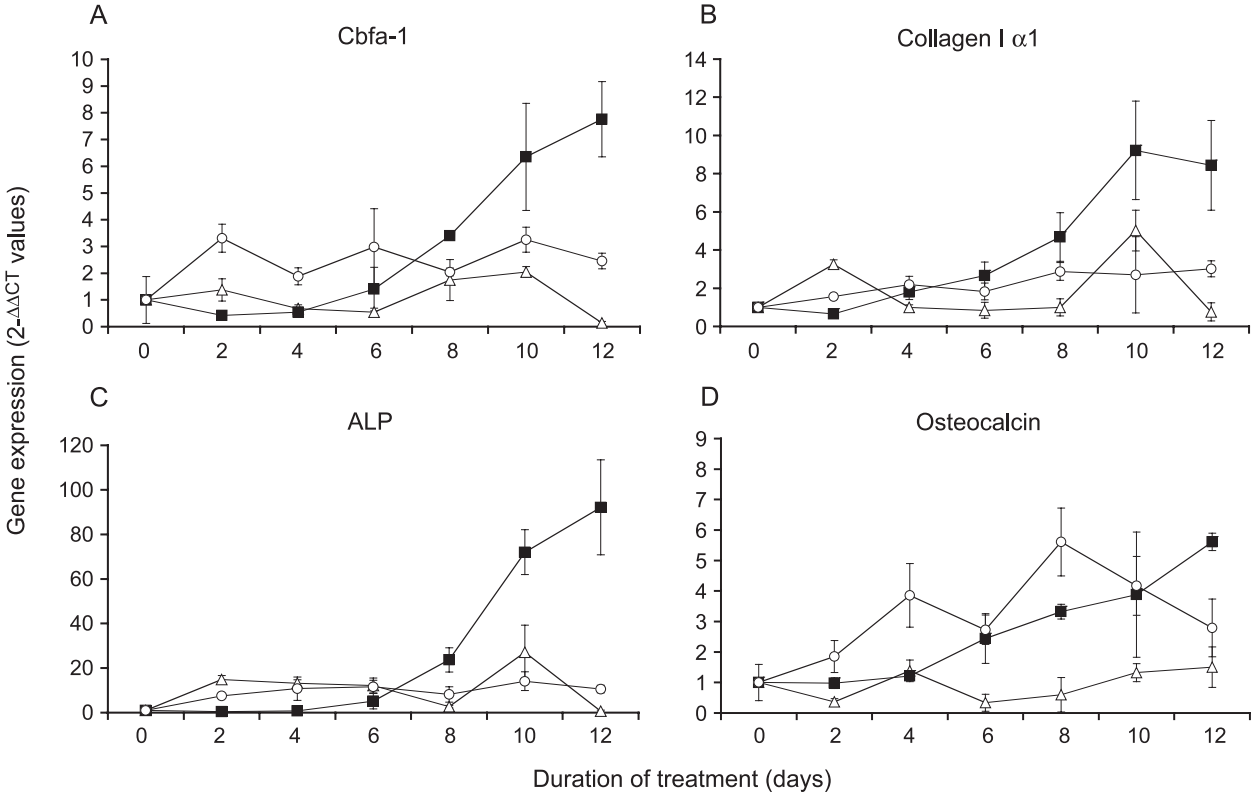

Fig. 2. Gene expression of cbfa-1 (A), α1 chain of type I collagen (B), alkaline phosphatase (C), and osteocalcin (D) quantified by RT–PCR after exposure to ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). Media were renewed every second day. The expression of fetal bone cells (■), adult bone cells (○), and mesenchymal stem cells (△) are shown as $2^{-\Delta\Delta CT}$ values, relative to their respective untreated group. Results are plotted as the mean ± SEM of three individual experiments.

The induction of proliferation with the full differentiation mix was significantly higher for the fetal bone cells than for the adult bone cells starting from day (d) 6 until the end of the experiment (Fig. 1A). Maximum difference was observed at day 12 (252.3% of the induction of adult bone cells, $P < 0.01$). For this parameter, there was no significant difference between the mesenchymal stem cells and the fetal bone cells treated with osteogenic factors.

Dexamethasone had a positive effect on proliferation of fetal bone cells (Fig. 1B). Compared to the minimal differentiation mix, it was significatively higher starting at day 8. This difference was observed with the same range until the end of the experiment (d8: +47%, $P < 0.05$; d10: +69%, $P < 0.0091$; d12: +68%, $P < 0.0022$). Conversely, the vitamin D_3 mix did not have a proliferative effect. When added together with dexamethasone, 1α,25-$(OH)_2D_3$ reduced the growth rate significantly at day 12, compared to dexamethasone alone (−24%, $P < 0.033$).

Gene expression

Results of relative gene expression obtained by real-time RT–PCR for cbfa-1, α1 chain of type I collagen, ALP, and osteocalcin are shown in Fig. 2 for human primary fetal bone cells in comparison with adult bone cells and mesenchymal stem cells. Cbfa-1 gene expression (Fig. 2A) of fetal bone cells started to increase at day 6 and reached a maximum at day 12 with a 3-fold higher upregulation than observed for the adult bone cells (315.9%; $P = 0.001$) and a 56-fold higher upregulation than obtained for the mesenchymal stem cells (5586.5%; $P = 0.002$) at this time point. Strongest upregulation of the α1 chain of type I collagen gene (Fig. 2B) was also observed for the fetal bone cells. At day 12, fetal bone cells showed a significantly higher level of gene expression than adult bone cells (279.5%; $P = 0.0112$) and mesenchymal stem cells (1108.7%; $P = 0.0022$). For the fetal bone cells, upregulation of the ALP gene (Fig. 2C) started at day 6 and increased continuously until day 12. At this time point, strongest differences in gene expression were observed with a 9-fold higher upregulation compared to adult bone cells (875%; $P = 0.0011$) and an even 140-fold higher expression than obtained for mesenchymal stem cells (13,635%; $P = 0.0006$). Osteocalcin gene expression (Fig. 2D) of fetal bone cells was induced slowly and late, with a significantly higher upregulation of the gene in fetal bone cells than in adult bone cells (201.2; $P = 0.0126$) and in mesenchymal stem cells (372.9%; $P = 0.0022$) at the end of the experiment.

Alkaline phosphatase activity

The induction of ALP enzymatic activity with the full differentiation mix was significantly higher for the mesenchymal stems cells than for the fetal and the adult bone cells starting from day 4 until the end of the experiment (Fig. 3A). Maximum difference was observed at day 6 (255.9% of the induction of fetal bone cells, $P < 0.001$, and 487.6% of adult bone cells induction, $P < 0.0003$). Compared with adult bone cells, fetal bone cells showed a higher induction of ALP enzymatic activity with a maximum difference at day 10 (222.2%, $P < 0.0007$). Detailed effects on ALP activity of 1α,25-$(OH)_2D_3$ and dexamethasone when added to ascorbic acid and β-glycerophosphate for fetal bone cells are shown in Fig. 3B. ALP enzymatic activity was enhanced in the group treated with the full differentiation mix, with a first significant difference comparing with the DMEM group at day 4 (d4: +322%, $P < 0.0179$; d6: +84%, $P < 0.0054$; d8: +530%, $P < 0.002$; d10: +183%, $P < 0.0001$; d12: +283%, $P < 0.0002$). The dexamethasone mix had also an increasing effect but starting at day 6 (d6: +81%, $P < 0.0067$; d8: +541%, $P < 0019$; d10: +246%, $P < 0.0001$; d12: +188% $P < 0.0042$). A comparable pattern was observed with adult bone cells, but the inductions were reduced comparatively to fetal bone cells (data not shown).

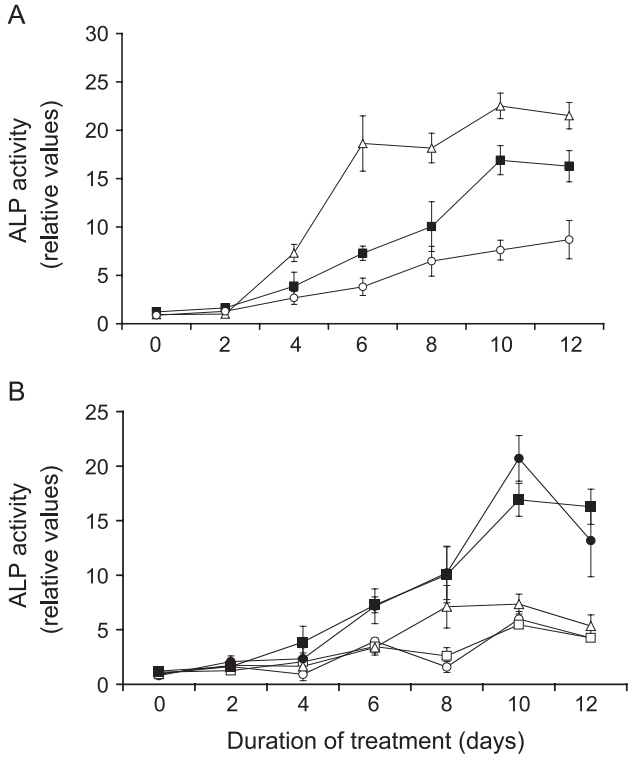

Fig. 3. (A) ALP enzymatic activity of fetal bone cells (■), adult bone cells (○), and mesenchymal stem cells (△). Cells were treated with ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). (B) ALP enzymatic activity of fetal bone cells treated with ascorbic acid 50 μg/ml and β-glycerophosphate 1 mM (minimal differentiation mix, □); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and dexamethasone 10 nM (dexamethasone mix, ●); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and 1α,25-$(OH)_2D_3$ 10 nM (vitamin D_3 mix, △); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix, ■). Ratios relative to the untreated group at day 0 are shown. Results are expressed as the mean ± SEM of three experiments performed in triplicate. Media were renewed every second day.

In vitro mineralization

First positive Von Kossa staining of fetal bone cells was obtained after 2 weeks of treatment with the full differentiation mix (Fig. 4A), as for MC3T3 E1 used as positive control (data not shown). In contrast, adult bone cells and mesenchymal stem cells did not show positive Von Kossa staining after 2 weeks of treatment, but after 5 and 3 weeks of treatment, respectively (data not shown). The same patterns as for the fetal cells were then obtained treating these cells for a longer period (data not shown). Further studies on fetal bone cells showed that dexamethasone did strongly increase in vitro calcium deposition, whereas 1α,25-$(OH)_2D_3$ did not show a comparable effect (Fig. 4B). Nodule formation was visible after the first week and strongly enhanced starting from the second week of treatment for fetal cells receiving the full differentiation mix (Fig. 5A), whereas no obvious difference was observed for adult cells at this time point (data not shown). Interestingly, fetal bone cells were able to constitute nodules even without any treatment of differentiation. When observed by scanning electron microscopy after 4 weeks of treatment, highly organized structures containing layers of cells trapped in an abundant collagenous matrix and aggregates were distinguishable in these nodules. Energy dispersive x-rays spectroscopy analysis did clearly identify the characteristic peaks of energy corresponding to Ca and P elements, indicating that in vitro mineralization occurred (Fig. 5B). Nodules were observed for adult cells starting from the fifth week and calcium phosphate crystal deposits 1 week later (data not shown).

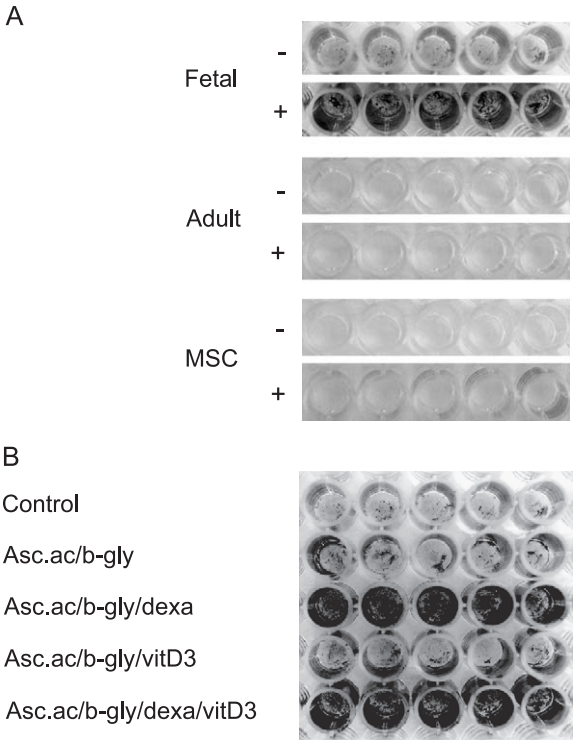

Fig. 4. (A) Von Kossa staining of the extracellular matrix of fetal bone cells, adult bone cells, and mesenchymal stem cells after 2 weeks of treatment. Control (−) and treated (+) groups are shown. Cells treated received ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). Media were renewed every second day. First positive stainings were observed after 3 and 5 weeks for mesenchymal cells and adult bone cells, respectively. At these time points, the groups treated with the full differentiation mix were markedly Von Kossa positive. (B) Von Kossa staining of fetal bone cells treated with ascorbic acid 50 μg/ml and β-glycerophosphate 1 mM (minimal differentiation mix); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and dexamethasone 10 nM (dexamethasone mix); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, and 1α,25-$(OH)_2D_3$ 10 nM (vitamin D_3 mix); ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). Media were renewed every second day.

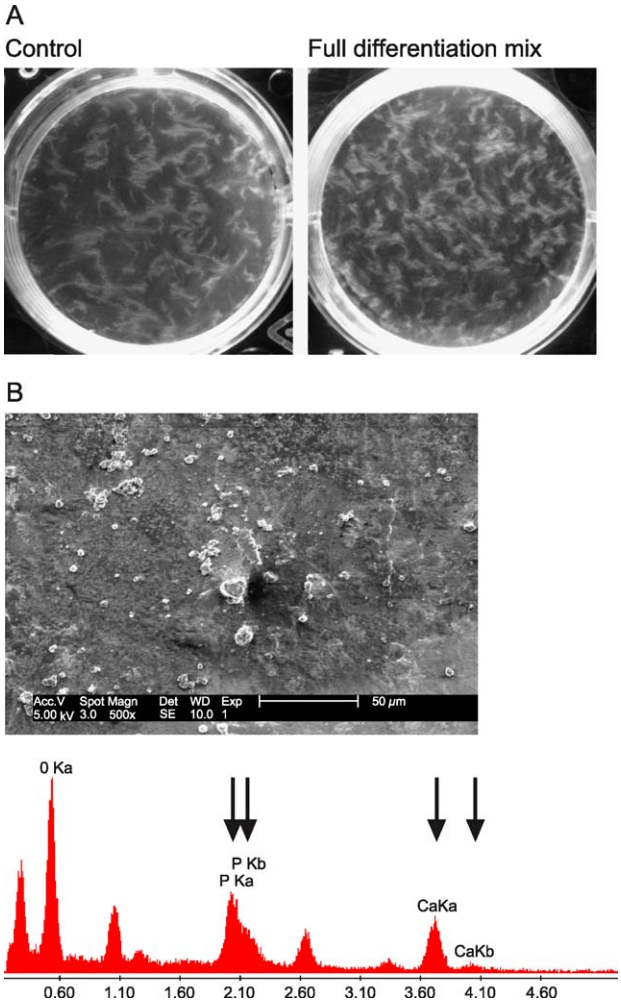

Fig. 5. (A) Bone-like nodule formation observed with fetal bone cells after 2 weeks. Control group and group treated with ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix) are shown. First nodules were detected in the control group after the first week, and their number was increasing with time. (B) Extracellular matrix of fetal bone cells after 4 weeks of treatment with ascorbic acid 50 μg/ml, β-glycerophosphate 1 mM, dexamethasone 10 nM, and 1α,25-$(OH)_2D_3$ 10 nM (full differentiation mix). SEM micrograph and SEM energy dispersive x-rays spectroscopy analysis are presented. Arrows indicate characteristic peaks of energy corresponding to Ca and P elements. Media were renewed every second day.

Discussion

The aim of this work was to investigate the osteoblastic potential of human primary fetal bone cells in vitro and to evaluate their possible use for tissue engineering. We first compared the behavior of bone fetal cells to adult bone and mesenchymal stem cells for their responsiveness to osteogenic factors. Then we characterized fetal bone cells in more details for their ability to be induced into the bone pathway. A key finding was the observation of spontaneous bone-like nodule formation after only 2 weeks and before adult cells and mesenchymal stem cells. Additionally, fetal bone cells showed a strong response to differentiation factors.

The growth rate of fetal bone cells was similar to mesenchymal stem cells, but faster than the growth rate of bone cells from a 31-year-old donor. The proliferative ability was even slower with cells obtained from older donors (Table 1). This age dependency was already observed and could be attributed to a decrease of proliferative precursor cells on trabecular bone surfaces [39]. However, age-dependent changes in the population of osteoprogenitor cells are discussed controversially. Recent results have given evidence of aging in marrow stromal progenitor cells [10,40–46]. Other reports demonstrated that osteogenic progenitor cells were maintained during aging [47–49]. The question of changes in the osteoprogenitor cell population with age is further complicated by the possibility of sex-dependent differences [50–52]. Age-related osteoponia may also depend from the skeletal site, depending to the variable trabecular–cortical bone ratio at different locations [53]. The proliferative aptitude is of major interest in the perspective to use human primary fetal bone cells for research or tissue engineering: a rapid expansion facilitates the creation of an important bank of cells, starting from one donor. Remarkably, the high capacity for self-renewal in vitro did correlate with telomerase activity for fetal cells, as demonstrated by the telomerase repeat amplification protocol assay (data not shown). In addition to its role in cell replication, telomerase is required for differentiation of murine bone marrow mesenchymal stem cells in vitro [54]. A possible mechanism leading to in vitro accelerated osteogenesis by telomerized bone marrow stromal stem cells could be attributed to the upregulation of the osteogenic genes cbfa-1, osteorix and osteocalcin [7]. The presence of ascorbic acid and β-glycerophosphate had no effect on proliferation. This observation was previously described for rat calvaria osteoblasts in cultures which maintained the same growth rate when β-glycerophosphate was added to ascorbic acid [28]. Dexamethasone accelerated the proliferation of both fetal and adult bone cells. When treated with dexamethasone or basic fibroblast growth factor, adult cells were proliferating faster but significantly slower than fetal cells from the untreated DMEM group (data not shown). Although $1\alpha,25\text{-}(OH)_2D_3$ did not have any effect on proliferation compared with the minimal differentiation mix, its presence slowed down the positive effect of dexamethasone. This was detected with fetal as well as with adult cells. The combining action of the two hormones was described to strongly increase the adipocyte population and their differentiation but to decrease the osteoblastic cell population in a long-term rat bone marrow culture [55]. Osteoblastic cell proliferation and differentiation were increased by dexamethasone and inhibited by $1\alpha,25\text{-}(OH)_2D_3$. Our findings are in accordance with these observations, and further studies are needed to address the issues concerning adipocyte differentiation.

During this proliferative sequence, genes involved in the production and deposition of extracellular matrix started to be expressed which denotes a strong osteogenic potential of primary fetal bone cells compared to adult and mesenchymal stem cells. The induction of cbfa-1 gene expression observed is essential for osteoblast function and differentiation. It is also a critical gene involved in bone matrix deposition [56]. Early upregulation of collagen Iα1 confirmed its induction before any other current matrix component and prior to ALP [28,57,58]. Fetal cell gene expression of ALP was augmented due to the presence of dexamethasone, $1\alpha,25\text{-}(OH)_2D_3$, ascorbic acid, and β-glycerophosphate. The time course of ALP gene expression roughly fits with the increase of activity of this enzyme. These results are consistent with previous studies where the regulation of ALP by glucocorticoids was ascertained at protein and mRNA levels [59–61]. Exposing the rat osteosarcoma cell line ROS 17/2.8 to dexamethasone, the rise in alkaline phosphatase mRNA levels could be attributed entirely to an increase in gene transcription [62]. The immortalized human fetal osteoblastic cell line hFOB/ER9 showed an increase of ALP activity during mineralization [63], whereas in rat fetal calvaria cultures, ALP was expressed maximally during the early stages of differentiation [28]. In our model, ALP activity of fetal bone cells reached a maximum activity after 10 days, when nodule formation started to be markedly enhanced. ALP enzymatic activity of mesenchymal stem cells was also strongly enhanced by osteogenic factors. Adult bone cells showed the same pattern for ALP enzymatic activity, with an earlier induction for the full differentiation mix, compared with the group receiving only dexamethasone additionally to ascorbic acid and β-glycerophosphate (data not shown). For adult cells, ALP enzymatic activity was observed to vary according to the age of the donor as previously demonstrated [64].

Osteocalcin, one of the most abundant noncollagenous proteins in bone, was also upregulated at the gene expression level, first in adult bone cells, and later in fetal bone cells. This was not observed with mesenchymal stem cells during the first 12 days of treatment, indicating that their extracellular matrix maturation was not as advanced as fetal cells. The delayed mineralization observed with mesenchymal stem cells in comparison with fetal cells could be due to their less differentiated phenotype. Corroborating this observation, osteocalcin gene expression

is controlled by cbfa-1 [31], and mesenchymal stem cells did not upregulate their cbfa-1 expression during the treatment. Although its biological function has not been precisely defined, specific interaction with hydroxyapatite was demonstrated [65]. New evidence indicates that osteocalcin participates in the regulation of mineralization and bone turnover [28]. A vitamin D regulatory element was identified in its promoter [66–69]. In most species, 1α,25-$(OH)_2D_3$ upregulates osteocalcin biosynthesis. Upregulation of osteocalcin indicated that fetal bone cells were fully differentiated into osteoblasts and started to mineralize. This was in accordance with the fact that in vitro calcium deposition was observed by Von Kossa staining after 2 weeks of treatment. Nodule formation increased gradually and was maximum at the end of the treatment (data not shown). Dexamethasone augmented the nodule number dramatically, when added to ascorbic acid and β-glycerophosphate. Comparable results were obtained with murine embryonic stem cells [70]. In primary rat calvaria cell cultures, additional nodule formation was observed, and the self-renewal capacity of the bone-nodule forming cells was increased [71]. This could be due to the induction of proliferation of more mature osteoblast precursors, enabling them to achieve sufficient numbers of cell divisions to form a visible nodule, or the effect targeted a separate subpopulation of progenitors requiring glucocorticoid to proliferate and/or differentiate [10,72]. The existence of subpopulations of osteoblasts that differs in their responsiveness to dexamethasone was previously suggested in clonal rat osteosarcoma cells [73].

Furthermore, characterization of the mineralized bone nodules by scanning electron microscopy clearly demonstrated that they were made of osteoblasts, extracellular collagen fibrils, and crystal deposits. Detailed analysis of the crystals by energy dispersive x-rays spectroscopy showed that these structures were containing Ca and P elements. Such a substratum produced by differentiating osteoblasts was described as an anchorage for calcifying collagen fibers synthesized by the same cells in a rat model [74]. Signs of mineralization are fundamental in perspective to use primary fetal bone cells for artificial bone synthesis.

For this study, the number of passages was arbitrarily fixed to 3. Similar results concerning fetal bone cells proliferation rate, ALP activity, and in vitro mineralization were obtained at passage 6 (data not shown), demonstrating that the multilineage capability was maintained.

In conclusion, our data support the idea that human primary fetal bone cells are able to differentiate into mature osteoblasts when stimulated. This full differentiation process displayed the appearance of specifically mineralized bone-like nodules. Due to their more rapid growing rate and their strong responsiveness to differentiation treatment compared with adult cells and mesenchymal stem cells, their use could be of great interest for fundamental research, pharmaceutical screenings, and especially for bone tissue engineering.

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