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Effect of Calcium-Infiltrated Hydroxyapatite Scaffolds on the Hematopoietic Fate of Human Umbilical Vein Endothelial Cells

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Human umbilical vein endothelial cells · Hemangioblast · Hematopoietic stem cells · Hydroxyapatite · Calcium

Abstract
Foamed hydroxyapatite offers a three-dimensional scaffold for the development of bone constructs, mimicking perfectly the in vivo bone structure. In vivo, calcium release at the surface is assumed to provide a locally increased gradient supporting the maintenance of the hematopoietic stem cells niche. We fabricated hydroxyapatite scaffolds with high surface calcium concentration by infiltration, and used human umbilical vein endothelial cells (HUVECs) as a model to study the effects on hematopoietic lineage direction. HUVECs are umbilical vein-derived and thus possess progenitor characteristics, with a prospective potential to give rise to hematopoietic lineages. HUVECs were cultured for long term on three-dimensional porous hydroxyapatite scaffolds, which were either infiltrated biphasic foams or untreated. Controls were cultured in two-dimensional dishes. The release of calcium into culture medium was determined, and cells were analyzed for typical hematopoietic and endothelial gene expressions, surface markers by flow cytometry, and hematopoietic potential using colony-forming unit assays. Our results indicate that the biphasic foams promoted a hematopoietic lineage direction of HUVECs, suggesting an improved in vivo-like scaffold for hematopoietic bone tissue engineering.

Introduction

Hemangioblasts are the bipotential angiohematopoietic stem cells that give rise to both hematopoietic stem cells (HSCs) and endothelial cells (ECs) [1]. The hypothesis that there is a common angiohematopoietic progenitor was developed by Sabin [2] more than 50 years ago, based on the close association of HSCs and vascular cell lineages [3, 4]. Indeed, primitive erythroblasts and ECs are known to develop from the extraembryonic mesoderm [5, 6]. Hemangioblasts have been isolated from mouse embryonic stem cells and have been shown to be capable of differentiating into both ECs and hematopoietic lineages [7, 8]. Cogle et al. [9] reported that human retinal neovascularization originates from human HSCs, which also suggests that HSCs have functional hemangioblast activity. Grant et al. [10] found that adult HSCs provided functional hemangioblast activity in a mouse

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model, producing both blood cells and ECs in neovascularization.

Human umbilical vein endothelial cells (HUVECs), derived from human umbilical veins [11], are a well-known source of primary ECs [12]. The isolated human endothelial progenitor cells (EPCs) from HUVECs express KDR, CD133, and CD34, as described by Mou et al. [13]. HUVECs are used as a model system of ECs to study the regulation of EC function, the mechanical response of ECs, the development of atherosclerosis, and angiogenesis [14]. Cells with characteristics of HUVECs can be isolated from immune-privileged fetal tissue [15], and express a high level of fetal immune privilege marker, CD95 (Fas) ligand [16]. Thus, HUVECs might have the capacity to differentiate into cells with an immune-cell character.

We hypothesized that there is a strong relationship between the HSC environment in vivo and the fate of hemangioblasts. If a cell population with a potential hemangioblast character, like HUVECs, is placed in a hematopoietic microenvironment, this could induce differentiation into hematopoietic cell lineages. In vivo, HSCs proliferate and reside in a microenvironment in the bone marrow, called the endosteal niche, close to the surface of the trabecular bone [17]. In order to induce hematopoietic differentiation of HUVECs, we deployed 3-dimensional (3D) hydroxyapatite (HA) scaffolds which simulate the architecture and chemical composition of in vivo bone. A calcium-rich HA scaffold with 90% porosity was used in the long-term culture to achieve a physical and chemical environment similar to the HSC endosteal niche. According to Adams et al. [18], there is an extremely high calcium (Ca) concentration near the endosteal niche, and a high ionic Ca (Ca^{2+}) concentration has been shown to affect the fate of HSCs. They reported that there are fewer HSCs in bone marrow and relatively more HSCs in circulation in a calcium receptor (CaR)-deficient mice model. In consequence, we added a soluble Ca-rich phase into the HA as a second phase to provide soluble Ca^{2+}, which can be released at the scaffold's surface during culturing. We investigated the effects of the scaffold and the Ca-rich second phase on the hematopoietic fate of HUVECs in long-term culture.

Materials and Methods

In this study, we cultured HUVECs on plain HA scaffolds and Ca-rich biphasic HA scaffolds, the latter being processed by the infiltration of Ca salts into the partially sintered scaffold followed by sintering to a high density. The effects of these scaffolds on the fate of HUVECs were compared to negative controls using no scaffolds.

Scaffold Preparation

The HA scaffold was prepared by a direct foaming process. A suspension was made by mixing deionized water with Ca-deficient HA (Sigma-Aldrich, MO, USA) powder with 30 vol.% solids. Ammonium polymethacrylate polyelectrolyte dispersant (Darvan C, RT Vanderbilt Co., Norwalk, CT, USA) was then added to the deionized water, and the pH was adjusted to 5.5 with hydrochloric acid (36.5–38.0%) (J.T.Baker, PA, USA). The suspension was subsequently mixed at 2,500 rpm for 20 min, and a cationic surfactant (benzethonium chloride, Sigma-Aldrich) was added and mixed at 2,500 rpm for 2 min. In the final step, a 10% volume of heptane was added to create an emulsion, by mixing at high speed for 2 min.

The emulsion was poured into a paper mold in an incubator with 60% humidity and kept for 1 h; subsequently, the humidity was decreased to 40% until the foam had fully dried. The samples were then sintered in air at 1,000°C to burn away the mold and partially sinter the HA foam while retaining open porosity in the struts. The Ca-rich biphasic HA foams were processed by infiltrating the partially sintered HA. The foams were infiltrated with a 2-mol/L solution of calcium nitrate for 24 h in an evacuated container to make sure all the air was removed from the partially sintered foam. The foams were then immersed in ammonia hydroxide solution at pH 12–13 for 1 h to precipitate calcium hydroxide and calcium carbonate within the struts of the foam and on the surface of the struts. After drying, the foam was fired at 900°C and fully sintered to high density at 1,300°C to achieve the biphasic HA and calcium oxide foam. Based on our previous work [19], the calcium oxide formed $CaCO_3$ when exposed to air, and this phase was detected by X-ray diffraction of the ground foam. The phase-pure HA foams were processed by the same heat treatments but without the infiltration of the Ca salt.

Scaffold Characterization

The HA and Ca-infiltrated HA (Ca-HA) scaffolds were coated with palladium using a sputter coater, and examined with an XL30 scanning electron microscope (Philips/FEI Company, Hillsboro, OR, USA). During scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS) was applied on the surface of the scaffolds for examination of the Ca/P ratio.

Cell Culture

HUVECs from the human vascular endothelium were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) at passage 15. They were precultured and passaged for the expansion of cell numbers to passage 20. They were then seeded into 24-well plates at a density of 5,000 cells/cm^2 in 0.5 mL of F12-K medium (ATCC), containing 10% fetal bovine serum (PAA Laboratories), and 1% antibiotic-antimycotic premix (Invitrogen) (100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL Amphotericin B). Culture conditions included a blank well for the negative control containing no scaffold, a plain HA scaffold, and a biphasic Ca-HA scaffold. Cells were cultured in an incubator at 37°C and a humidified atmosphere of 95% air and 5% CO_2. The F12-K culture medium was replaced every 2–3 days. At 0, 5, 15, and 42 days, cells were harvested for gene expression analyses, fluorescence-activated cell sorting (FACS), and colony-forming unit (CFU) assay.

Gene Expression Analysis

Gene expression analyses were performed on preseeded HUVECs (day 0, control) and HUVECs harvested from cultures after day 5, 15, and 42 by real-time reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted from the cell lysate using an AllPrep DNA/RNA mini-kit (Qiagen, Valencia, CA, USA). The extracted RNA was reverse-transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Inventoried TaqMan probes (Applied Biosystems) were used to quantify gene expression for β-actin (a housekeeping gene used as an internal normalizer), CD31, CD144, CD34, CD45, and von Willebrand factor (vWF, Life Technologies). The StepOnePlus real-time PCR system was used, and data were collected with StepOne software v2.0 (Life Technologies). A negative PCR no-template control included water.

Flow Cytometry

Fresh HUVECs and HUVECs cultured for 5, 15, and 42 days were analyzed for their surface marker expression profiles by FACS. HUVECs at day 0 were also analyzed as the control sample. Cells were rinsed twice with PBS (without Ca and Mg), and after detaching with EDTA-trypsin (Life Technologies), single cells were resuspended and incubated with blocking buffer containing a 20% FcR block (Miltenyi, San Diego, CA, USA), 0.5% BSA, and 2 mM EDTA (Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (DPBS) without Ca and Mg (Life Technologies), pH 7.2. Controls included nonstained cells, and cells incubated with corresponding isotype controls (Becton Dickinson, Bedford, MA, USA). For surface marker staining, cells were incubated with fluorochrome-conjugated antibodies, an FITC-lineage cocktail (Lin 1, i.e., CD3, CD16, CD19, CD20, CD14, and CD56), PerCPCy5-CD34, BV421-CD31, PE-CD235a, APC-H7-CD45, AF647-CD309 (KDR), and AF700-CD38 (all Becton Dickinson). Additional fluorochrome-conjugated antibodies were BV510-CD19, PE-CD34, FITC-CD133, and a CD3/CD4/CD8a monoclonal antibody cocktail (APC-CD8a, FITC-CD4, and PerCP-eFluor 710-CD3) (all eBioscience, San Diego, CA, USA).

Compensation beads (Becton Dickinson) were used to compensate for potential spectral fluorochrome overlaps. Cells were analyzed with a FACS Aria II (Becton Dickinson). Raw data were analyzed with FlowJo software v9.5.2 (Tree Star, Ashland, OR, USA); a forward- versus side-scatter gate was applied to exclude cell debris and cell doublets.

Live Cell Sorting

HUVECs that had been cultured for 15 days were sorted for those cells that expressed HSC markers, defined as Lin–/CD34+/CD38–. Cells were rinsed twice with DPBS (without Ca and Mg), and after detaching with EDTA-trypsin (Life Technologies) or TryplE (Life Technologies), single cells were resuspended and incubated with blocking buffer containing a 20% FcR block (Miltenyi), 0.5% BSA, and 2mM EDTA (Sigma-Aldrich) in DPBS (without Ca and Mg, pH 7.2). Controls included nonstained cells. For the staining of surface markers, cells were incubated with fluorochrome-conjugated antibodies, FITC-Lin 1, AF700-CD38 (Becton Dickinson), PE-CD34 (eBioscience), and Sytox Blue dead cell stain (Invitrogen Detection Technologies, Eugene, OR, USA). The HSC gate-sorted cells were cultured for 5 days in HSC-specific culture medium containing 1% StemSpan CC100 (STEMCELL Technologies), and the 1% antibiotic-antimycotic premix (Invitrogen).

CFU Assay

We used the CFU assay to investigate the potential of HUVECs to form colonies of cells of hematopoietic lineages. Day 0 HUVECs and cultured HUVECs from day 15 and day 42 were placed in ultra-low-adherence Petri dishes in a complete MethoCult methylcellulose-based assay according to the manufacturer's instructions (STEMCELL Technologies) for 14 days. In addition, cultured and subsequently sorted cells expressing HSC markers (as described under "Live Cell Sorting" above) were assayed under the same conditions. Colonies were observed by phase microscopy (Invertoskop C, Carl Zeiss, Jena, Germany) and counted. Four different types of colonies were identified and their frequencies were compared to expected numbers (i.e., supplied by the manufacturer). These included: CFU-E (colony forming unit – erythrocyte), BFU-E (burst forming unit – erythrocyte), CFU-GM (colony forming unit – granulocytes, macrophage) and CFU-GEMM (colony forming unit – granulocyte, erythrocyte, macrophage, megakaryocyte).

Measurement of Medium Calcium Ion Concentrations

The Ca^{2+} concentrations were measured from the medium samples to detect the Ca released from the scaffolds over the culture period. All concentrations were measured using a Cobas b 221 blood analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Statistical Analysis

The data are given as means from 3 biological repeats ± standard deviation (SD). One-way ANOVA with the Tukey test was used to analyze statistical significance, with $p \leq 0.05$ considered significant.

Results

Characterization of the HA Scaffolds

In this long-term HUVEC culture, the HA and Ca-HA scaffolds provided a 3D structure for cell growth and proliferation. As the Ca-HA scaffolds were produced based on HA scaffolds, there is no observable difference between the architecture of these 2 kinds of scaffolds. SEM images of the open-porous structure of the scaffolds are shown in Figure 1. Each piece of scaffold has high porosity, with large, connected pores. The total porosity in the scaffolds was approximately 90%, with open porosities of approximately 87%, as reported previously [20]. Using EDS (Fig. 1c), the Ca/P ratio was approximately 1.70 at certain positions, while the stoichiometric Ca/P ratio for HA is 1.67. The Ca/P ratio for the whole surface area was approximately 1.57 (Fig. 1d), which indicates that the scaffold was HA-deficient.

Measurement of Calcium Ion Concentrations

During the 42 days of culture, we monitored the Ca^{2+} concentrations in the culture medium (Fig. 2). Whereas cultures with infiltrated HA had a significantly higher

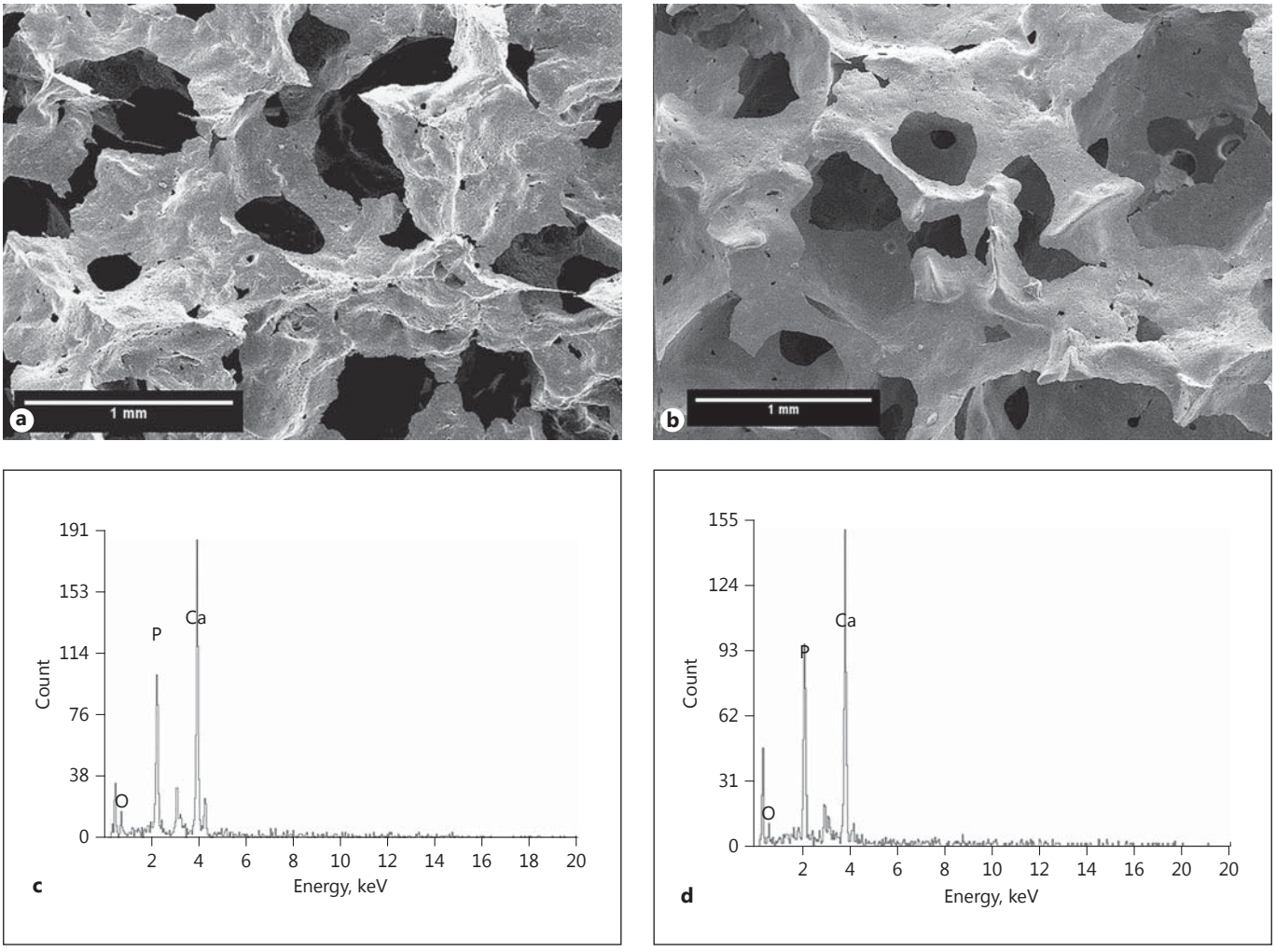

Fig. 1. a, **b** SEM images of high-porosity scaffolds implemented for HUVEC culture. **a** HA scaffold. **b** Infiltrated HA scaffold. **c** EDS result for the Ca-rich position on the infiltrated HA scaffold. **d** EDS result for the surface area on the infiltrated HA scaffold.

Ca^{2+} concentration than control cultures without a scaffold, cultures with noninfiltrated HA demonstrated a Ca^{2+} concentration similar to in the controls. The cultures with infiltrated HA gave a burst of high Ca^{2+} concentration for the first 3 days, probably due to the rapid dissolution of the Ca-rich ($CaCO_3$) second phase. The Ca^{2+} concentration decreased after 3 days of culture in the infiltrated samples, but the Ca^{2+} release was still maintained above that of the control cultures and the pure HA scaffold over the entire 40 days.

Gene Expression Analyses

Gene expression by the HUVECs was analyzed by RT-PCR (Fig. 3a–c) on days 5, 15, and 42 of culture. Results were normalized to freshly isolated day 0 cell samples. We analyzed genes typical for mature endothelium (*VWF* coding for vWF), endothelial progenitor cells and mature endothelium (*PECAM1* coding for surface CD31), HSCs, and endothelial progenitors (*CD34* coding for surface CD34), and mature hematopoietic cells (*PTPRC* coding for surface CD45).

After 5 days in culture, there was no significant difference between the 3 conditions in terms of gene expression s for CD34, CD45, and CD31; expression of CD45 was not detected at all. vWF expression was significantly higher in the control condition than in the scaffold conditions. However, this significant difference for vWF expression was absent at days 15 and 42, and it increased for

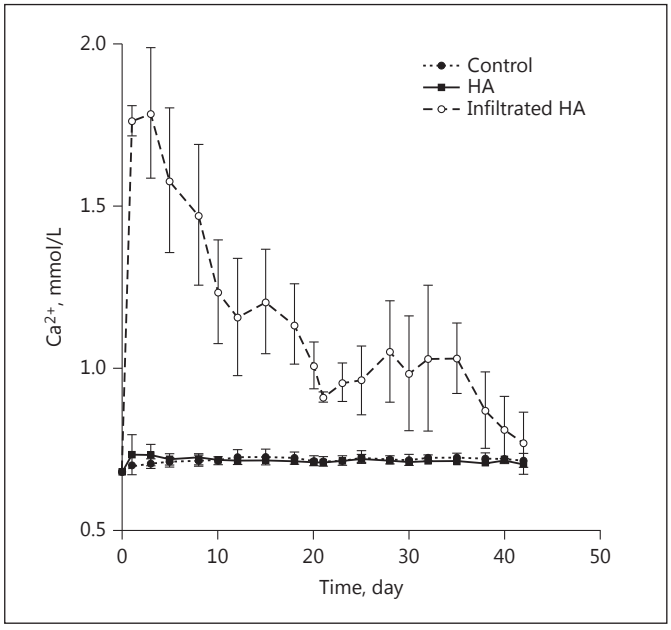

Fig. 2. Measurement of Ca^{2+} concentration in cell culture media over 42 days of culture. Data are given as means ± standard deviation from 3 biological repeats.

all 3 conditions over time. After 15 days, CD34 expression of the control was statistical significantly higher than that of cultures with scaffolds. After 42 days, the CD34 expression of the control was still higher than that of cultures with scaffolds, but was not statistically significant. CD45 expression was detectable from day 15 to day 42, and cultures with scaffolds demonstrated significantly higher expression than the control without scaffold at both time points; at day 15, cultures on Ca-HA scaffolds showed significantly higher CD45 expression than those on non-infiltrated HA scaffolds. Throughout culture, CD31 expression was maintained, and there was no difference between culture conditions.

Flow Cytometry

Based on our data on increased CD45 gene expression in cultures with HA scaffolds, we were specifically interested in investigating the hematopoietic lineage differentiation potential of HUVECs. We analyzed percentages of HUVECs at 2 time points, day 15 and day 42, when the mature hematopoietic marker CD45 first appeared. The antibodies we used included CD235a for erythrocytes, CD45 for mature hematopoietic cells, a hematopoietic Lin 1 for mature lineage cells (including T lymphocytes, B lymphocytes, natural killer lymphocytes, monocytes, neutrophils, eosinophils, granulocytes, and macro-

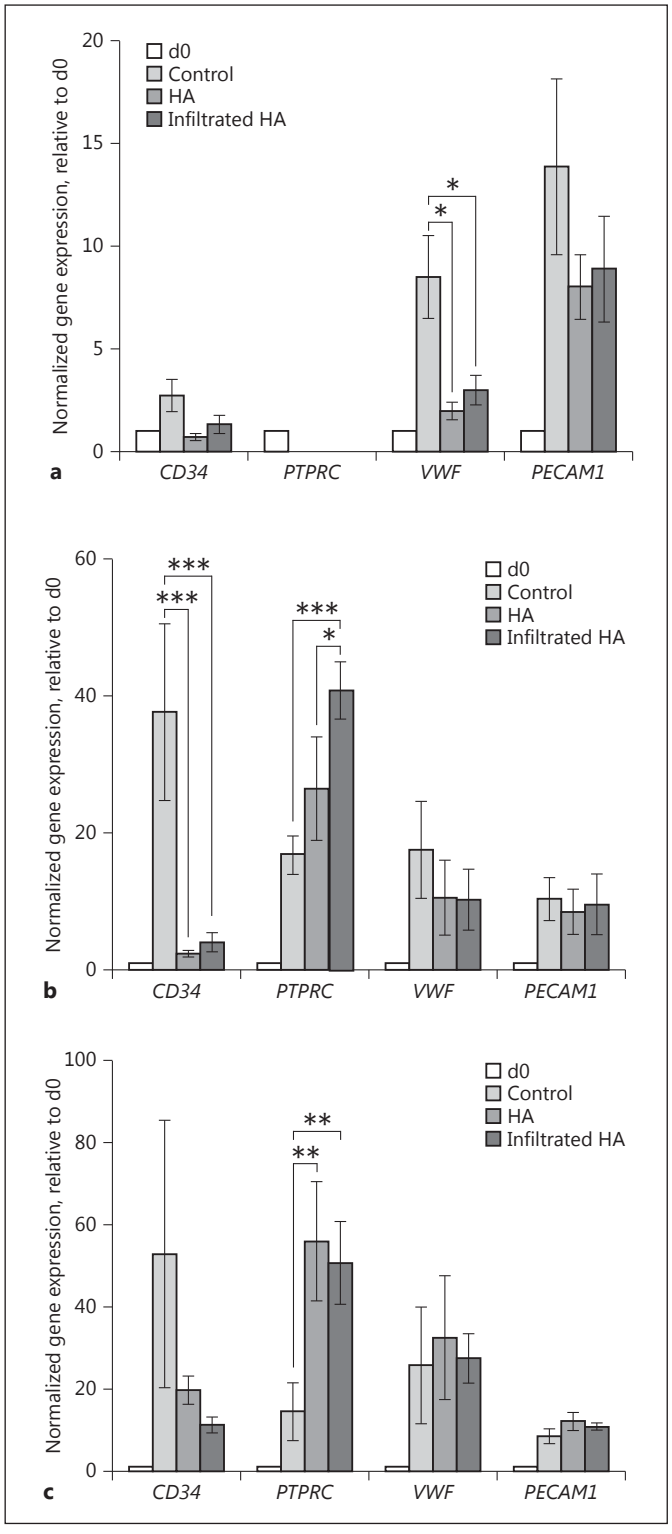

Fig. 3. RT-PCR result for *CD34*, *PTPRC* (CD45), *VWF* (vWF), and *PECAM1* (CD31) gene expression on day 5 (**a**), day 15 (**b**), and day 42 (**c**). d0, day 0. Data are given as means ± standard deviation. Statistical significant differences were *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; the Student *t* test.

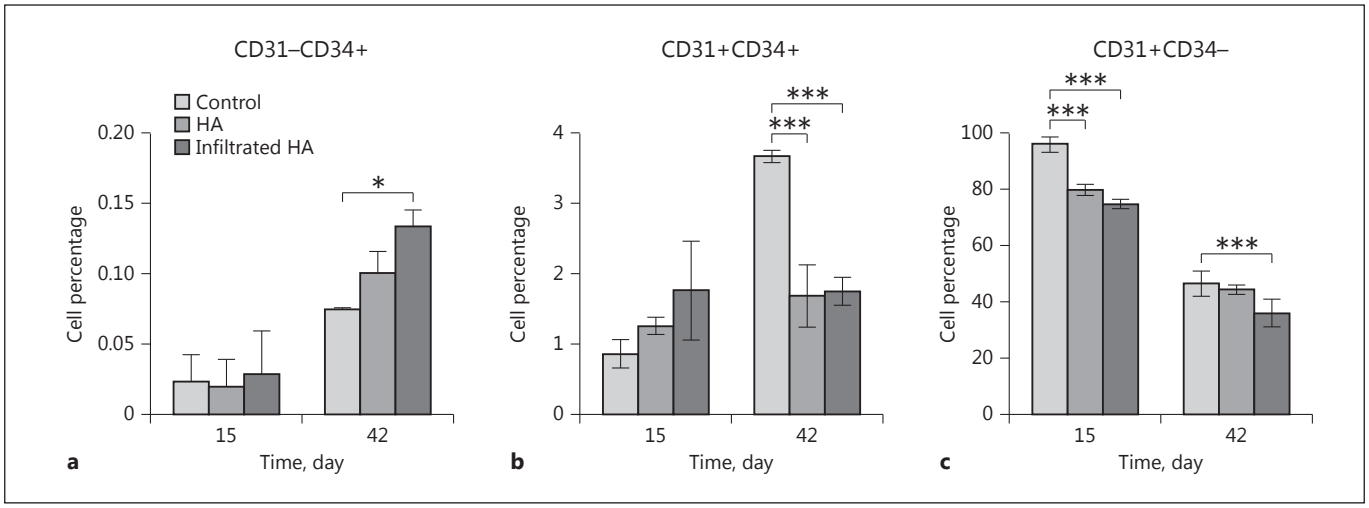

Fig. 4. FACS analyses of CD31–CD34+ hematopoietic progenitor cells (**a**), CD31+CD34+ endothelial progenitor cells (**b**), and CD31+CD34– mature endothelial cells (**c**) of HUVECs cultured on scaffolds of Ca-infiltrated hydroxyapatite (HA), plain HA, and controls without scaffolds after 15 and 42 days. Data are given as means ± standard deviation from 3 biological repeats. *** $p < 0.001$, * $p < 0.05$; statistically significant differences.

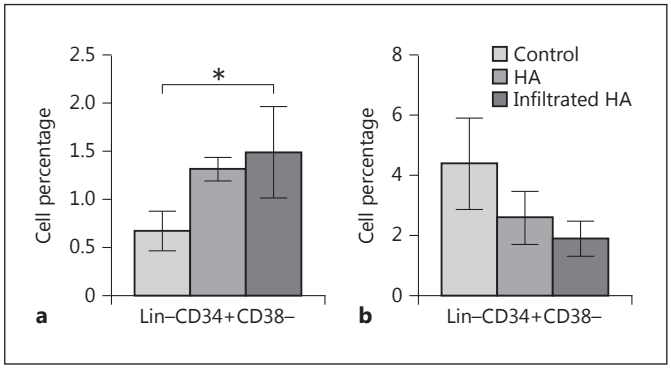

Fig. 5. FACS analyses of Lin–CD34+CD38– hematopoietic stem cells of HUVECs cultured on scaffolds of infiltrated hydroxyapatite (HA), plain HA, and controls without scaffolds after 15 (**a**) and 42 (**b**) days. Data are given as means ± standard deviation from 3 biological repeats. * $p < 0.05$; statistically significant difference.

phages), CD34, and CD31, in combination, for the detection of several hematopoietic/endothelial cell types (including CD31–CD34+ for hematopoietic progenitor cells, CD31+CD34+ for endothelial progenitor cells and hemangioblasts, and CD31+CD34– for mature ECs), and a combination of Lin–CD34+CD38– for HSCs.

We observed a significant increase of CD31–CD34+ hematopoietic progenitors (Fig. 4a) when HUVECs were cultured on infiltrated HA (but not on plain HA), after a long-term culture of 42 days. In addition, the percentages of CD31+CD34+ endothelial progenitors (Fig. 4b) were significantly lower in cultures with HA (both infiltrated and plain HA) when compared to controls. The percentages of CD31+CD34– ECs (Fig. 4c) were significantly lower in cultures with HA; this was the case at both time points examined (days 15 and 42) for infiltrated HA, but only on day 15 for plain HA.

Percentages of HSCs, defined as Lin–CD34+CD38– cells (Fig. 5), in the shorter-term culture of 15 days (Fig. 5a) were initially lower in controls than on the HA scaffolds, but had increased by 42 days of culture (Fig. 5b), from approximately 0.67 to 4.39%. In comparison, the percentages of HSCs on both types of scaffolds remained stable throughout culturing (i.e., on days 15 and 42).

We also investigated the expression of mature hematopoietic surface markers, including CD45, CD235a, and various hematopoietic lineages (Lin 1) (Fig. 6). After 15 days of culture (Fig. 6a), the percentages of cells on HA scaffolds expressing CD235a and Lin increased compared to controls, with a statistically significant higher percentage of Lin+ cells observed on the Ca-HA scaffold. By 42 days of culture (Fig. 6b), the percentages of cells expressing hematopoietic markers including Lin, CD45, and CD235a were significantly higher on the Ca-HA scaffold than in the control without scaffolds; in addition, CD45 expression was significantly higher on the Ca-infiltrated scaffolds than on the plain scaffolds. Cells cultured on the

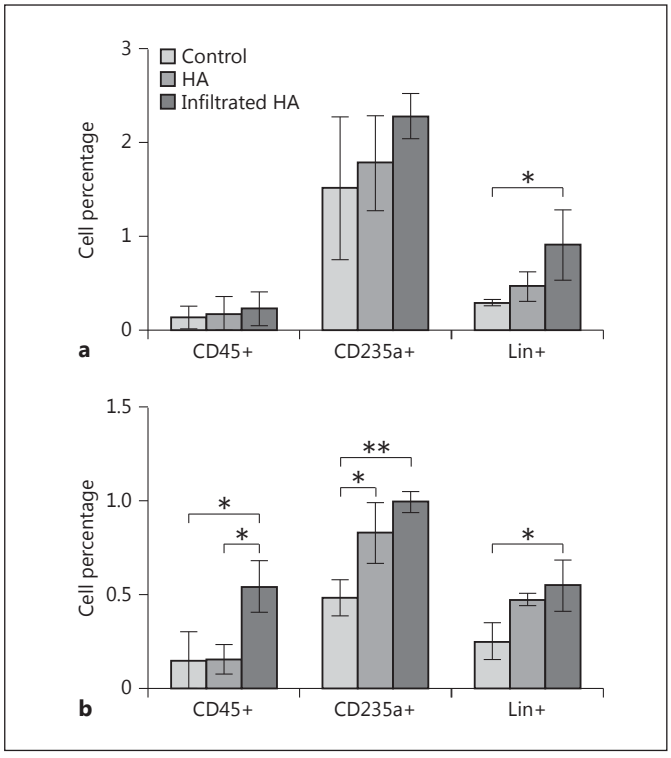

Fig. 6. FACS analyses of CD45+ hematopoietic cells, CD235a+ erythrocytes, and Lin+ cells of HUVECs cultured on scaffolds of infiltrated hydroxyapatite (HA), plain HA, and controls without scaffolds after 15 (**a**) and 42 (**b**) days. Data are given as means ± standard deviation from 3 biological repeats. ** $p < 0.01$, * $p < 0.05$; statistically significant differences.

plain HA scaffolds also demonstrated significantly higher expression of CD45 and CD235a than controls without scaffolds.

We further investigated the expression of the characteristic T cell antigens, CD3, CD4, and CD8a, and the B cell antigen, CD19 (Fig. 7a). On day 0, HUVECs did not express any T cell or B cell markers, which indicated that there were no mature hematopoietic cells present. By day 15, we could detect hematopoietic cells in the HA and Ca-HA conditions. The percentages of cells expressing mature hematopoietic cell markers (CD3, CD4, and CD19) were significantly higher in the HA condition than in the controls. In the Ca-HA condition, more cells expressed the T cell markers CD3 and CD4 than the cells in the control condition without scaffolds did. Compared to day 0 samples, on day 15, the percentage of CD31–/CD34+/CD133+/KDR+ cells, that characterizes hemangioblasts, had sharply decreased from 0.65 to 0.02% in all 3 conditions (Fig. 7b). However, cells cultured on both HA and Ca-HA scaffolds had a higher percentage of CD31–/CD34+/CD133+/KDR+ cells than the control. There was no significant difference between the HA and the Ca-HA conditions.

Table 1. CFU assay of HUVECs at all time points

Sample	CFU-E	BFU-E	CFU-GM	CFU-GEMM
Day 0	0	0	0	0
Day 15 control	0	0	0	0
Day 15 HA	0.67±0.67	0	0.67±0.67	0
Day 15 Ca-HA	0.67±0.67	0	0.67±0.67	0
Day 42 control	0	0	0	0
Day 42 HA	0.67±0.67	0	1.00±1.00	0
Day 42 Ca-HA	0.67±0.67	0	0.33±0.33	0

Fresh cells on day 0 and cells that had been cultured for 15 or 42 days in control conditions (no scaffold), on plain hydroxyapatite (HA) scaffold, or calcium-infiltrated HA (Ca-HA) scaffold were cultured for 14 days in methylcellulose-based hematopoietic assay medium. Colony types were counted microscopically, and data are given as means ± standard deviation from 3 biological repeats.

Live Cell Sorting

We also sorted cells from HUVEC cultures that expressed characteristic HSC markers (Lin–/CD34+/CD38–). The percentages of the selected cells were 0.7% in the controls, 1.0% in the HA condition, and 1.1% in the Ca-HA condition.

CFU Assay

In order to investigate the possibility that HUVECs have the hematopoietic progenitor features to form colonies of various hematopoietic lineages, we performed CFU assays (Table 1). HUVECs without culturing were assayed as on day 0 and did not show any colony formation. HUVECs cultured for 15 or 42 days without scaffolds (control) did not form colonies either, indicating that HUVECs in conventional culture do not have any hematopoietic colony-forming potential. HUVECs that had been cultured for 15 or 42 days on Ca-HA and plain HA scaffolds, however, demonstrated the formation of colonies. The presence of CFU-E and CFU-GM in the assay proved the existence of functional hematopoietic progenitors in HA and Ca-HA conditions after 15 and 42 days of culture.

In addition, in order to investigate the hematopoietic functional potential of hematopoietic-oriented cells,

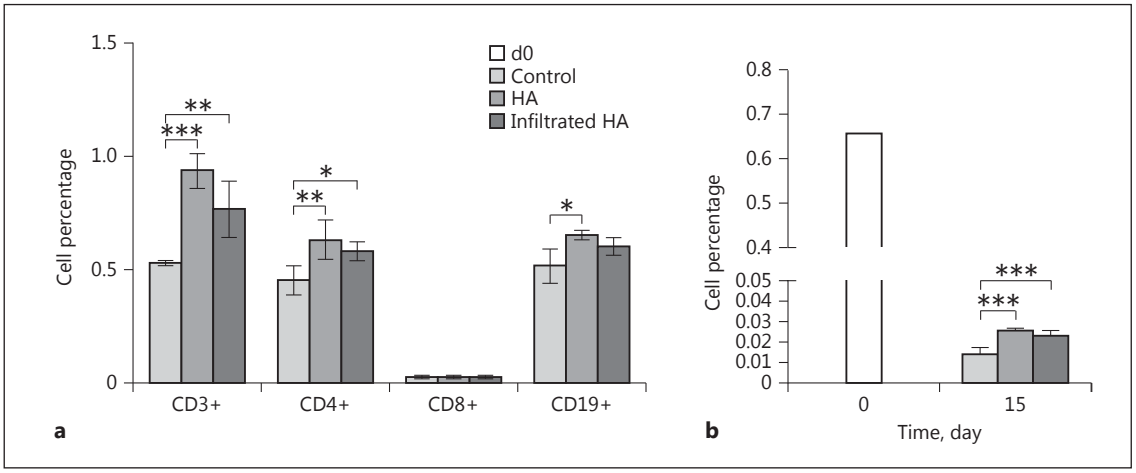

Fig. 7. a FACS analyses of CD3+, CD4+, CD8+, and CD19+ hematopoietic cells of HUVECs cultured on scaffolds of infiltrated hydroxyapatite (HA), plain HA, and controls without scaffolds after 15 days in culture. **b** CD31–CD34+CD133+KDR+ expression characterizing hemangioblasts within fresh HUVECs and HUVECs cultured on scaffolds of infiltrated hydroxyapatite (HA), plain HA, and controls without scaffolds after 15 days. d0, day 0. Data are given as means ± standard deviation from 3 biological repeats. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; statistically significant differences.

Table 2. CFU assay of HUVECs on day 15

Day-15 sample	CFU-E	BFU-E	CFU-GM	CFU-GEMM
Control	0	0	0	0
HA	4.33±1.67	0	6.67±0.33	0
Ca-HA	6.00±2.00	0	8.00±1.00	0

Cells that had been cultured for 15 days without a scaffold (control conditions), on a plain hydroxyapatite (HA) scaffold, or a calcium-infiltrated HA (Ca-HA) scaffold were selected for their expression of the HSC markers Lin–/CD34+/CD38–, and cultured in HSC medium for 5 days. Subsequently, the harvested cells were cultured for 14 days in methylcellulose-based hematopoietic assay medium. Colony types were counted microscopically, and data are given as means ± standard deviation from 3 biological repeats.

CFU assays were performed with HSC marker-sorted cells that were subjected to a 5-day culture period in HSC medium (Table 2). Interestingly, no colonies could be found in the control condition (no scaffolds), but CFU-E and CFU-GM colonies were detected in both scaffold cultures (noninfiltrated and Ca-HA scaffolds). The differences between the controls and the HA or Ca-HA conditions were significant. However, there was no significant difference between the HA and the Ca-HA condition.

Discussion

In our research, we performed long-term culture of HUVECs for 6 weeks to mimic important aspects of the endosteal niche microenvironment in bone marrow, making use of 3D HA-based calcium phosphate scaffolds. We hypothesized that simulating basic aspects of the endosteal niche, including the 3D architecture and the ability to release Ca^{2+}, could promote hematopoietic differentiation. In long-term culture of HUVECs, cells with characteristics of HSCs appeared, and more cells expressing mature hematopoietic markers were present at the end of the culture. Cells were also tested in terms of their functionality and potential using the CFU assay; the results demonstrated that at least a fraction of the cells were able to differentiate into erythrocytes and form macrophages.

In HSC in vitro culture, ECs have been shown to be an important component for maintenance of the HSC niche [21], perhaps due to the hemangioblast which has been described as a common precursor for endothelial and hematopoietic cells during development [3, 7, 22–25] as well as in the adult [26–28]. Definitive proof of the existence of adult hemangioblasts was provided by discovering adult HSCs that had functional hemangioblast activity during retinal neovascularization [10]. CD34+ adult hemangioblasts have also been discovered in bone marrow and peripheral blood [29–32]; however, it is not yet

understood how chemical and physical signals (e.g., the presence of Ca or the 3D environment) can contribute to hematopoietic lineage differentiation of ECs. During early embryonic development, the hemogenic endothelium can give rise to hematopoietic cell types (review [33]). The hemogenic endothelium has been defined as displaying an endothelial phenotype and morphology, and having the capacity to form hematopoietic offspring and endothelial tubules/sheets in culture [34] (review [33]). The overlap in the expression of hematopoietic and endothelial markers for vascular endothelial cadherin, CD31, CD34, and CD45 suggests a close developmental relationship between hematopoietic cells and ECs [35–37] (review [33]).

In our work, we found that HUVECs, which are commonly used as a mature EC lineage model, obtained HSC functionality after long-term culture. A low percentage of HUVECs with hemangioblast characteristics could be detected, as determined by CD31–/CD34+/CD133+/KDR+ expression. The significantly higher percentage of cells expressing markers of mature hematopoietic cells (erythrocyte marker CD235a, lineage markers, and monocyte marker CD45) in scaffold culture than in the controls indicates that ECs can be stimulated into the hematopoietic lineage by culture on HA scaffolds. Some of the cells also expressed lymphocyte marker CD4. In addition, the CFU activity of cells that had been cultured on HA scaffolds (but not those that had been cultured in 2-dimensional Petri dishes) suggests that HA scaffolds promote the hematopoietic lineage direction. HUVECS, which were sorted for HSC markers after culture on scaffolds, demonstrated an enhanced hematopoietic potential. HA scaffolds have been applied in tissue engineering [38, 39] and stem cell culture [40–43], commonly for bone tissue engineering. For example, osteogenic differentiation of mesenchymal stem cells has been reported, using HA alginate scaffolds [43] or HA nanoparticles [44]. In addition the long-term maintenance of bone marrow-derived HSCs was achieved in bioreactors with HA scaffolds [45].

Calcium is known to be important for stem cell differentiation and proliferation [46]. In HSCs, different calcium receptors have been described (CaR [18] and GPCRs [47]). These receptors are required to maintain HSCs near the endosteal surface of the bone [48], indicating that HSC maintenance is sensitive to Ca^{2+}. We found that Ca-HA scaffolds induced HUVECs to a hematopoietic fate, suggesting that the local surface Ca^{2+} concentration might play an important role in the hematopoietic differentiation of ECs.

Conclusion

In this study, we investigated the effects of 3D, porous, Ca-HA scaffolds on HUVECs in long-term culture. In culture on scaffolds, some HUVECs acquired hematopoietic characteristics. HA scaffolds per se increased percentages of cells positive for erythrocyte markers and CFU activity, with Ca infiltration significantly increasing the percentages of CD45+ and hematopoietic lineage marker-positive cells. These results indicate that high-porosity Ca-HA scaffolds support the hematopoietic lineage direction of HUVECs, suggesting that HA infiltrated with Ca provides an improved in vivo-like scaffold for hematopoietic bone marrow tissue engineering.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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