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Endogenous Collagen Influences Differentiation of Human Multipotent Mesenchymal Stromal Cells

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Human multipotent mesenchymal stromal cells (hMSCs) are multipotent cells that, in the presence of appropriate stimuli, can differentiate into different lineages such as the osteogenic, chondrogenic, and adipogenic lineages. In the presence of ascorbic acid, MSCs secrete an extracellular matrix mainly composed of collagen type I. Here we assessed the potential role of endogenous collagen synthesis in hMSC differentiation and stem cell maintenance. We observed a sharp reduction in proliferation rate of hMSCs in the absence of ascorbic acid, concomitant with a reduction in osteogenesis *in vitro* and bone formation *in vivo*. In line with a positive role for collagen type I in osteogenesis, gene expression profiling of hMSCs cultured in the absence of ascorbic acid demonstrated increased expression of genes involved in adipogenesis and chondrogenesis and a reduction in expression of osteogenic genes. We also observed that matrix remodeling and anti-osteoclastogenic signals were high in the presence of ascorbic acid. The presence of collagen type I during the expansion phase of hMSCs did not affect their osteogenic and adipogenic differentiation potential. In conclusion, the collagenous matrix supports both proliferation and differentiation of osteogenic hMSCs but, on the other hand, presents signals stimulating matrix remodeling and inhibiting osteoclastogenesis.

Introduction

TISSUE ENGINEERING FOCUSES on the restoration, maintenance, or improvement of diseased or damaged tissues.^{1–3} Currently, when a tissue is damaged or lost, autografts or synthetic prostheses are used in an attempt to restore its function. Nevertheless, problems such as multiple surgeries, insufficient material availability, risk of infection, failure of graft materials, and rejection by the host are commonly associated with these treatments. Despite these drawbacks, several tissue-engineered products have found their way into the clinic.^{4,5} Presently, research is focused on the use of autologous cells to improve integration of grafts as well as to enhance the biological efficacy of cell-based therapies.^{6,7} Over the past decade, there has been much interest in the use of human multipotent mesenchymal stromal cells (hMSCs) in tissue engineering. hMSCs are multipotent cells that are able to differentiate, depending on the stimulus, into several lineages, including the osteogenic, chondrogenic, and adipogenic lineages *in vitro*.^{8,9} Because these cells can be easily isolated from bone marrow aspirates and several other sources, and expanded *in vitro*, they can be used for various cell-based therapeutic approaches such as tissue engineering and cell therapy, and can serve as cytokine and growth factor

factories.¹⁰ In stem cell biology, the growth factor milieu is very important for maintenance of stemness. For example, propagation of undifferentiated pluripotent mouse embryonic stem cells is dependent on the cytokine leukemia inhibitory factor.¹¹ Similarly, during *in vitro* culture of hMSCs, basic fibroblast growth factor (bFGF) is used to enhance their growth rate and to select for a subpopulation of hMSCs with a more extensive expansion potential.¹² Growth factors and other diffusible molecules, such as dexamethasone (dex), cyclic adenosine 3',5'-monophosphate, 1,25-dihydroxy-vitaminD, and bone morphogenetic protein 2 (BMP-2), are also used to drive osteogenic differentiation of hMSCs.^{13–15} On the other hand, the presence of extracellular matrix (ECM) proteins can be crucial for maintenance of stemness and differentiation. For instance, the tendon stem cell niche is dependent on the presence of biglycan and fibromodulin, which highlights the instructive potential of these molecules in addition to their well-known structural role as ECM proteins.^{16,17}

hMSCs are typically expanded in the presence of ascorbic acid, which plays a key role in the synthesis of ECM.¹⁸ Ascorbic acid is an essential cofactor for the enzymes prolyl and lysyl hydroxylase, which hydroxylate proline and lysine residues, respectively, during posttranslational modifications of collagen.¹⁹ As such, hMSCs are expanded in the presence

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of a matrix mainly composed of collagen type I. Collagen type I is also the most abundant protein in bone ECM, and no collagen is synthesized in the absence of ascorbic acid.²⁰ It has been reported that addition of ascorbic acid to the culture medium greatly enhanced the growth rate and extended the lifespan of hMSCs.^{21,22} In addition, ascorbic acid has been shown to promote differentiation and mineralization of MC3T3-E1 preosteoblast cells by enhancing the accumulation of mature collagen in ECM.^{23–25} ECM also plays an important role during chondrogenic differentiation. During this process, cells change from the characteristic fibroblast-like morphology to large round cells surrounded by abundant ECM, due to a dynamic reorganization of the cytoskeletal network.²⁶ This ECM is a dense connective tissue, consisting of a highly organized network of collagen (mostly type II) and large aggregating proteoglycans.²⁶ ECM components play a key role in stem cell niches and have been shown to support hematopoiesis by means of controlling the pool of hematopoietic progenitor cells.^{27–29}

Moreover, the instructive properties of ECM can vary depending on the structure of collagen. Parameters such as the level of crosslinking, conformation of the molecule, and ECM compliance can control differentiation into a given lineage.^{30–32} The first steps toward the use of ECM-inspired tissue-engineered applications have been taken. For example, using decellularization methods Ott *et al.* were able to preserve cardiac architecture and ECM components, key elements to engineer a functional heart.³³ ECM-derived matrices have been shown to control cell fate and stem cell maintenance. Chen *et al.*³⁴ showed that bone-marrow-derived ECM plays a critical role in maintenance of stemness. Postovit *et al.* elegantly showed that ECM derived from embryonic stem cells could control the behavior of cancer cells by decreasing their invasiveness.³⁵

Based on the known instructive role of ECM in some contexts, it can be expected that the matrix deposited by culture-expanded hMSCs has an effect on the stemness and differentiation potential of the cells growing on it.

In this study we investigated the effect of the collagenous matrix deposited during expansion of hMSCs. We confirmed the positive effect of collagen type I on osteogenic differentiation of hMSCs, and we investigated whether the presence of collagen type I during the expansion phase subsequently interferes with adipogenic, osteogenic, and chondrogenic differentiation.

Materials and Methods

Cell culturing

Bone marrow aspirates (5–15 mL) were obtained from patients who had given written informed consent. hMSCs were isolated and proliferated as described previously, unless stated otherwise.³⁶ Briefly, aspirates were resuspended using a 20-gauge needle, plated at a density of 5×10^5 cells/cm², and cultured in the MSC proliferation medium, which contains α -minimal essential medium (Life Technologies, Gaithersburg, MD), 10% fetal bovine serum (FBS; Life Technologies), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ascorbic acid; Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/mL penicillin (Life Technologies), 100 μ g/mL streptomycin (Life Technologies), and 1 ng/mL bFGF (Instruchemie, Delfzijl, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO₂. The medium was refreshed

twice per week, and cells were used for further subculturing or cryopreservation on reaching near confluence. The hMSC basic medium was composed of the hMSC proliferation medium without bFGF; the hMSC osteogenic medium (dex) was composed of the hMSC basic medium supplemented with 10^{-8} M dex (Sigma, St. Louis, MO); the hMSC mineralization medium (min) was composed of a basic medium supplemented with 10^{-8} M dex and 0.01 M β -glycerophosphate (BGP; Sigma).

Cell proliferation assay

Cell proliferation was assessed using an Alamar blue assay according to the manufacturer's protocol. Briefly, the culture medium was replaced with the medium containing 10% Alamar blue solution (Biosource, Camarillo, CA), and cells were incubated at 37°C for 4 h. Fluorescence was measured at 590 nm on a (Perkin Elmer, Wellesley, MA) LS50B plate reader.

Alkaline phosphatase flow cytometry

To analyze the role of ascorbic acid in alkaline phosphatase (ALP) expression of hMSCs, cells were grown in the presence or absence of different concentrations of ascorbic acid in a basic or in an osteogenic medium.

After 7 days of culture, cells were trypsinized and incubated for 30 min in block buffer (phosphate-buffered saline [PBS] with 5% bovine serum albumin [BSA; Sigma] and 0.05% NaN₂) and in PBS with 1% BSA plus primary antibody (anti-ALP B4-78; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) for 30 min or with isotype control antibody (mouse anti-human immunoglobulin G1; BD Biosciences, Breda, The Netherlands). Cells were then washed three times with washing buffer (PBS with 1% BSA and 0.025% NaN₂) and incubated with secondary antibody (rat anti-mouse immunoglobulin G1 phycoerythrin (PE); BD Biosciences) for 30 min. Cells were washed two times and resuspended in 300 μ L of washing buffer plus 10 μ L of Viaprobe (BD Biosciences) for live/dead cell staining. Only living cells were used for further analysis. ALP expression levels were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Mineralization and calcium assay

For mineralization experiments, hMSCs were seeded in triplicate at 5000 cells/cm² in T25 culture flasks in a basic (containing BGP) or mineralization medium, either in the presence or in the absence of ascorbic acid. The total calcium deposition was assayed using a calcium assay kit (Sigma Diagnostics, St. Louis, MO) according to manufacturer's protocol. Briefly, the culture medium was aspirated, and cells were washed twice with calcium- and magnesium-free PBS (Life Technologies) and incubated overnight with 0.5 N HCl on an orbital shaker at room temperature. The supernatant was collected, and the calcium content was measured at 575 nm (Lambda 40; Perkin Elmer).

Adipogenic assay

hMSCs were seeded in triplicate at 25,000 cells/well in 24-wells plates and grown in the adipogenic medium con-

taining Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA), 10% FBS, 100 U/mL penicillin with 100 µg/mL streptomycin, 0.2 mM Indomethacin (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), 10^{-6} M dex, and 10 µg/mL human insulin (Sigma). As a negative control, DMEM with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin was used. After 21 days, cells were fixed for at least 4 h in 0.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck, Darmstadt, Germany) in 45 mL MilliQ and 5 mL of 4% formaldehyde (Sigma), rinsed with water, incubated for 5 min in 60% isopropanol (Sigma), and stained for 5 min in freshly filtered (0.22 µm) Oil Red O solution (3 mg/mL in 60% isopropanol; Sigma). After rinsing with demineralized water, pictures of the stained cells were taken using a Nikon Digital Sight DS-Fi1 camera, Melville, NY.

Chondrogenic assay

To test the chondrogenic potential of hMSCs, 200,000 cells/well were seeded in round-bottom 96-wells plates and pelleted by centrifugation. The chondrogenic medium consisted of DMEM supplemented with 40 µg/mL proline (Sigma), 50 µg/mL insulin transferrin selenium-premix (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.2 mM ascorbic acid, 100 µg/mL sodium pyruvate (Gibco), 10 ng/mL transforming growth factor beta 3 (TGF-β3) (R&D Systems, Abingdon, United Kingdom), 10^{-7} M dex, and 500 ng/mL BMP-6 (kindly provided by Genera Incorporated, Zagreb, Croatia). After 21 days, pellets were fixed with cold formalin for histological analysis, dehydrated, embedded into paraffin, and stained with alcian blue 8GX (Sigma). For analysis of glycosaminoglycans, pellets were digested with 1 mg/mL proteinase K (Sigma) and lysed, and absorbance was measured after adding 1,9-dimethylmethylene blue chloride solution in PBE buffer (14.2 g/L Na_2HPO_4 and 3.72 g/L Na_2EDTA , pH 6.5; Sigma). To correct for cell number, Cyquant cell proliferation assay kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions.

Collagen biochemical analysis

We determined the collagen content and the ratio hydroxyproline/proline (Hyp/Pro) as a measure of collagenous to noncollagenous proteins. To determine the collagen content, hMSCs were cultured for 21 days in the presence or absence of ascorbic acid, or alternatively in the presence of 1 mM L-2-azetidine carboxylic acid (AZC), either in the basic or in the osteogenic medium. After 21 days, samples were hydrolyzed (110°C, 20–24 h) with 750 µL 6 M HCl, dried, and redissolved in 800 µL of water containing 2.4 mM homo-arginine (internal standard for amino acids; Sigma). Samples were diluted 100-fold with 0.1 M sodium borate buffer (pH 8.0) for amino acid analysis. Derivation and subsequent chromatography of the amino acids was performed as described.^{37,38} Collagen content was calculated from the total amount of hydroxyproline in each sample, assuming 300 hydroxyproline residues per collagen molecule and a molecular weight of collagen of 300 kD.

Microarray analysis

To analyze the effects of ascorbic acid in the gene expression profile of hMSCs, we seeded 5000 cells/cm² in T25

flasks either in the basic or in the osteogenic medium, in the presence or absence of ascorbic acid, for 10 days.

Total RNA was isolated using an RNeasy mini kit (Qiagen, Venlo, The Netherlands) and on-column DNase treated with 10 U RNase-free DNase I (Gibco) at 37°C for 30 min. DNase was inactivated at 72°C for 15 min. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry.

Affymetrix 900649 GeneChip®, Santa Clara, CA, Human Exon 1.0 ST Arrays were hybridized, and the fluorescence intensity was normalized by dividing the fluorescence intensity of each sample by the medium intensity of the array. The fold induction reflects the ratio between the fluorescence intensity in the first condition versus the fluorescence intensity of the second condition. We generated four different lists (genes upregulated and downregulated in basic medium vs. basic medium without ascorbic acid, and genes upregulated and downregulated in osteogenic medium vs. osteogenic medium without ascorbic acid). Only genes co-expressed in the two media were considered for further analysis. DAVID, an online annotation tool, was used for identification of enriched functional groups within gene lists.³⁹

In vivo bone formation

To evaluate the effect of ascorbic acid on *in vivo* bone formation by hMSCs, we seeded 2×10^5 cells onto three porous biphasic calcium phosphate ceramic scaffolds of approximately 2–3 mm, containing 20% β-tricalcium phosphate and 80% hydroxyapatite sintered at 1300°C, prepared in-house. Cells were cultured *in vitro* for 14 days in the osteogenic medium in the presence or absence of ascorbic acid.

To analyze if the absence of ascorbic acid had an effect on proliferation when cells were grown on the scaffolds for 14 days, a DNA assay was performed to quantify differences between the two conditions. Briefly, cells were seeded as described above and lysed at day 14 using a 0.1% Triton X-100 solution in PBS. DNA was quantified using the cyQuantGR dye by measuring the fluorescence at 520 nm.

Before implantation, the tissue-engineered constructs were washed with PBS. Six immunodeficient mice (HsdCpb:NRI-nu; Harlan, Horst, The Netherlands) were anesthetized using isoflurane. The surgical sites were cleaned with ethanol, subcutaneous pockets were created, and three particles were implanted. After 6 weeks, the implants were retrieved and fixed in 0.14 M cacodylic acid buffer pH 7.3 containing 1.5% glutaraldehyde. The fixed samples were dehydrated in ethanol series and embedded in methyl methacrylate. Sections were processed on a histological diamond saw (Leica SP1600, Wetzlar, Germany) and stained with 1% methylene blue (Sigma) and 0.3% basic fuchsin solution (Sigma). At least five sections per scaffold were imaged, and the percentage of bone per scaffold area was calculated using a personal computer (PC)-based system with KS400 software (version 3; Zeiss, Hamburg, Germany). All experiments were approved by the local animal care and use committee.

Statistical analysis

The data were analyzed using Student's paired *t*-test or a one-way analysis of variance followed by a Tukey's multiple comparison test ($p < 0.05$), and values represent the mean and standard deviation of three independent measurements.

Results

Effects of ascorbic acid on endogenous collagen formation

We quantified the amount of collagen deposited by hMSCs after 21 days of culture in the presence or absence of ascorbic acid, either in the basic or in the osteogenic medium. In the absence of ascorbic acid, the collagen content was strongly reduced both in the basic and osteogenic medium (1000 and 200-fold, respectively) in comparison with the medium containing ascorbic acid. No statistically significant difference could be observed in the amount of collagen deposited by cells grown in the basic or osteogenic medium (209 ± 71 vs. 212 ± 4 μ g of collagen per sample, respectively; Fig. 1A). We further analyzed the ratio of collagenous to noncollagenous proteins (expressed as the Hyp/Pro ratio) in the presence or absence of ascorbic acid both in the basic and osteogenic medium. In the absence of ascorbic acid, there was a significant reduction in this ratio, independently of the medium (20-fold reduction in the basic medium and 44-fold reduction in the osteogenic medium; Fig. 1A), demonstrating a decrease in the amount of collagenous proteins synthesized in the absence of ascorbic acid. We also observed a significant difference in the Hyp/Pro ratio between the basic and osteogenic medium, which suggests a shift in protein synthesis upon osteogenic differentiation (Fig. 1A).

Effect of collagen on *in vitro* proliferation of hMSCs

We analyzed the effect of collagen deposition during expansion of hMSCs. There was a marked difference in cell morphology between cells grown in the presence or absence of ascorbic acid (Fig. 1B). The most striking difference between hMSCs grown in the two conditions was the ap-

pearance of stress fibers in the absence of ascorbic acid, a feature observed even when cells were allowed to grow in the presence of ascorbic acid for the first 3 days upon which ascorbic acid was removed. To analyze the effect of collagen on the efficiency of hMSC isolation, we plated bone marrow aspirates either in the presence or absence of ascorbic acid. Cells grew until one of the conditions reached near confluence, upon which they were trypsinized and the number of cells was calculated. We observed a marked decrease in the number of cells in the absence of ascorbic acid for three out of four donors tested (Fig. 1C). Because the number of colony-forming unit-Fs/mL of bone marrow was not determined, we cannot exclude that the increase in cell number in the presence of ascorbic acid is due to higher cloning efficiency. To investigate a potential effect on proliferation, we analyzed the effect of a range of concentrations of ascorbic acid on proliferation of hMSCs. Figure 1D clearly shows that addition of ascorbic acid, independent of the medium or concentration tested, resulted in enhanced proliferation of hMSCs. The effect was dose dependent and reached a plateau at a concentration of 0.2 mM, which is the concentration typically used in the hMSC expansion medium.

Ectopic bone formation by hMSCs in the absence of ascorbic acid

In ectopic bone formation models, hMSCs are expanded *in vitro* in the presence of ascorbic acid, but the beneficial effect of its presence for the *in vivo* bone-forming capacity is not known. Here, we investigated the *in vivo* bone-forming capacity of hMSCs grown *in vitro* for 14 days on ceramic scaffolds, in the presence or absence of ascorbic acid. First, we analyzed the number of cells on the scaffolds after 14 days of *in vitro* culture. Figure 2A shows a 35% reduction

FIG. 1. The absence of ascorbic acid inhibited collagen deposition and altered cell morphology and proliferation (A). Collagen content and Hyp/Pro ratio (ratio between collagen/noncollagenous proteins) in hMSCs grown for 21 days in the basic or osteogenic medium in the presence or absence of ascorbic acid. Note that in the absence of ascorbic acid, hardly any collagen could be detected in the samples $*p < 0.05$ (B). hMSCs were cultured for 21 days in the presence or absence of ascorbic acid. Note that cells grown in the absence of ascorbic acid showed marked differences in morphology with the control (C). Bone marrow cells were

obtained from various donors and the hMSC yield after first passage was calculated. Note that in the absence of ascorbic acid there was a decrease in the number of hMSCs for all the donors except one (donor 3). (D) Proliferation of hMSCs cultured in the osteogenic medium for 7 days in the presence of increasing concentrations of ascorbic acid. Note the increase in proliferation of hMSCs when ascorbic acid was present, independently of the concentration. Hyp/Pro, hydroxyproline/proline; hMSCs, human multipotent mesenchymal stromal cells; AA, ascorbic acid; Bas, basic medium; Dex, dexamethasone $*p < 0.05$. Color images available online at www.liebertonline.com/ten.

FIG. 2. Effect of ascorbic acid on *in vivo* bone formation by hMSCs (A). hMSCs were cultured for 14 days on biphasic calcium phosphate 1300 scaffolds in the presence or absence of ascorbic acid, and the DNA content was calculated. Note the significant decrease in DNA content when ascorbic acid was absent from the culture medium $*p < 0.05$. (B) hMSCs were cultured *in vitro* for 14 days on biphasic calcium phosphate 1300 scaffolds in osteogenic medium, with or without ascorbic acid. The constructs were implanted subcutaneously in immune-deficient mice for 6 weeks, and after explantation they were stained for newly formed bone with basic Fuchsin. Note the decrease in the percentage of bone per scaffold area in the absence of ascorbic acid $*p < 0.05$. (C) Representative histological sections showing newly formed bone 6 weeks after implantation.

tation. Note the difference in the amount of bone between hMSCs exposed to ascorbic acid (dex) relative to hMSCs cultured *in vitro* in the absence of ascorbic acid (dex-AA). The black area in the picture represents the scaffold and the arrow indicates newly formed bone. dex, dexamethasone, AA, ascorbic acid. Color images available online at www.liebertonline.com/ten.

in the number of cells on the scaffolds when cultured in the absence of ascorbic acid. Second, we analyzed the *in vivo* bone-forming potential of these cells upon subcutaneous implantation in immune-deficient mice. Six weeks after implantation, $4 \pm 1.5\%$ of bone tissue per scaffold area was observed in the case of hMSCs grown in the presence of ascorbic acid, whereas only $2 \pm 2.5\%$ of bone tissue per scaffold area was observed when cells were grown in the absence of ascorbic acid (Fig. 2B), clearly demonstrating the positive effect of ascorbic acid on ectopic bone formation by hMSCs.

Effect of ascorbic acid in the osteogenic program of hMSCs

Based on the previous results showing changes in morphology and a decrease in *in vivo* bone formation, we analyzed the effect of collagen on the osteogenic program of hMSCs. Dex-induced ALP expression of hMSCs, an early

marker of osteogenic differentiation, was significantly reduced in the absence of ascorbic acid (40% reduction in the percentage of ALP-positive cells between cells grown in osteogenic medium with vs. without ascorbic acid), indicating that the osteogenic program was impaired early in the differentiation cascade (Fig. 3A). Moreover, calcium accumulation by hMSCs, a phenotypical late marker of osteogenic differentiation, showed an almost complete inhibition of calcium accumulation in the absence of ascorbic acid, further indicating that also the later stages of osteogenic differentiation were impaired in its absence (Fig. 3B).

Effect of ascorbic acid on the gene expression profile of hMSCs

Next, we analyzed genome-wide gene expression in the presence or absence of collagen. We performed a microarray analysis on hMSCs cultured for 10 days either in the basic or

FIG. 3. The absence of ascorbic acid impairs osteogenic differentiation of hMSCs. (A) ALP expression by hMSCs cultured in the basic or osteogenic medium, in the presence or absence of ascorbic acid, for 7 days. Note the induction of ALP when cells were exposed to dex and a significant decrease in the percentage of ALP-positive cells in the absence of ascorbic acid. (B) Calcium accumulation by hMSCs

cultured in basic (with β -glycerophosphate) or mineralization medium, in the presence or absence of ascorbic acid, for 21 days. Note that in the absence of ascorbic acid, hardly any calcium could be detected in mineralization medium. ALP, alkaline phosphatase; AA, ascorbic acid.

in the osteogenic medium, in the presence or absence of ascorbic acid. Some of the changes in gene expression reflected our earlier observations. For instance, we observed that cells grown in the presence of ascorbic acid were enriched for genes related to the cell cycle and mitosis (see Supplemental Table S1, available online at www.liebertonline.com/ten). Further, genes involved in osteogenesis such as ALP were highly expressed in the presence of ascorbic acid. In contrast, noggin, a BMP antagonist, was upregulated in the absence of ascorbic acid. Interestingly, the proteases cathepsin K and cathepsin S were expressed higher in the presence of ascorbic acid, independent of the medium (5.9-fold in basic vs. basic-ascorbic acid, and 6.5-fold in osteogenic vs. osteogenic-ascorbic acid for cathepsin K, two fold for cathepsin S). In addition, several interferon-induced genes were upregulated upon exposure to ascorbic acid, suggesting that interferon signaling is enhanced in the presence of a collagenous matrix.

Expression of some genes was lower in the presence of ascorbic acid, which represents a group of genes suppressed by the presence of collagen. For instance, we observed lower levels of expression of TGF- β 2, aggrecan, hyaluronan synthase 1, and cartilage oligomeric matrix protein, all known to be involved in chondrogenic differentiation. CCAAT/enhancer binding protein gamma expression, a gene involved in adipogenic differentiation, was also lower in the presence of ascorbic acid. Expression of genes with an endothelial signature, such as endothelial differentiation lyso-phosphatidic acid G-protein-coupled receptor 7 and vascular endothelial growth factor A, was highest in the absence of ascorbic acid (a list of all the genes regulated can be found in Supplemental Table S1).

Absence of ascorbic acid and its effects on cell fate decision

Our results show that by adding ascorbic acid to the culture medium, we enhance the proliferation of hMSCs and differentiation into the osteogenic lineage, but, by doing so, we may compromise differentiation into other lineages. To confirm this, we expanded hMSCs, in the presence or ab-

sence of ascorbic acid, and differentiated them into the osteogenic, adipogenic, and chondrogenic lineages. When cells were expanded in the absence of ascorbic acid, their osteogenic potential was not significantly affected in two of the four donors tested, whereas a positive effect was seen in two other donors (Fig. 4A). When cells were exposed to an adipogenic differentiation protocol, we observed an increase in the number of Oil Red O-positive cells when cells were expanded in the absence of ascorbic acid in hMSCs from three donors (Fig. 4B). In the case of chondrogenic differentiation of hMSCs we observed that two out of four donors did not undergo chondrogenic differentiation irrespective of the presence or absence of ascorbic acid (data not shown). Moreover, the two donors that did differentiate into the chondrogenic lineage gave opposite results. In one case the expansion in the absence of ascorbic acid resulted in an increase in the amount of glycosaminoglycans per DNA, whereas in the other case there was a significant reduction (Fig. 4C).

The expansion of hMSCs in the absence of ascorbic acid can eventually select a cell population with a distinct differentiation potential in detriment of another cell population. Therefore, we characterized the cells using a panel of markers commonly used to characterize MSCs. Phenotypical characterization of both populations showed that we are using an MSC population and that the expansion in the presence or absence of ascorbic acid did not result in the selection of a specific population (Fig. 5).

To confirm that the effect of ascorbic acid on MSCs is solely due to the absence of endogenous collagen production and not due to another potential role of ascorbic acid, we used the known collagen inhibitor (AZC). We grew the cells in the osteogenic medium or osteogenic medium with AZC. Figure 6 shows that the presence of AZC significantly decreased the proliferation of hMSCs and calcium accumulation but had no effect on ALP expression (Fig. 6A–C). The less pronounced effect might be because of an incomplete inhibition of collagen production by AZC. Indeed, our results showed that AZC could only partially inhibit collagen production, but not to the same extent as depletion of ascorbic acid, which might explain the observed effects.

FIG. 4. Multilineage potential of hMSCs upon expansion in the presence or absence of ascorbic acid $*p < 0.05$. (A) Calcium accumulation by hMSCs expanded in the presence (++) or absence (-+) of ascorbic acid and cultured for 21 days in the mineralization medium. Note that hMSCs expanded in the absence of ascorbic acid showed a slight increase in calcium accumulation although not statistically significant (with the exception of D1 and D2) $*p < 0.05$. (B) Adipogenic differentiation of hMSCs expanded in the presence or absence of ascorbic acid. Note that cells expanded in the absence of ascorbic acid showed an increase in the number of Oil red O-positive cells. (C) Chondrogenic differentiation of hMSCs expanded in the presence or absence of ascorbic acid. Figure shows GAG/DNA quantification and the respective histological analysis (Alcian blue staining). GAG, glycosaminoglycans; Cond ++, cells expanded in the presence of ascorbic acid; Cond -+, cells expanded in the absence of ascorbic acid; AA, ascorbic acid. $*p < 0.05$. Color images available online at www.liebertonline.com/ten.

FIG. 5. Phenotypical characterization of hMSCs expanded in the presence or absence of ascorbic acid. Note that both cell populations express typical markers of MSCs and that the expansion in the absence of ascorbic acid did not change phenotypical expression.

Discussion

Soluble factors are typically used in the culture medium to drive differentiation of hMSCs. For example, dex, ascorbic acid, and BGP are used for osteogenic differentiation; TGF- β for chondrogenic differentiation; and indomethacin, isobutylmethylxanthine, and insulin for adipogenic differentiation.⁸

During osteogenic differentiation, hMSCs undergo a step-wise process leading to the deposition of an ECM, mainly composed of collagen type I, which will be mineralized in a later stage.⁴⁰ Ascorbic acid is an essential cofactor for the activity of a key enzyme involved in collagen synthesis—prolyl hydroxylase.⁴¹ In the absence of ascorbic acid, collagen deposition by the cells was drastically reduced, resulting in impaired osteogenic differentiation and proliferation. For

FIG. 6. Collagen inhibitors and osteogenic differentiation of hMSCs. (A) Proliferation of hMSCs was significantly inhibited in the presence of AZC, independently of the medium $*p < 0.05$. (B) Culture of hMSCs for 7 days in the presence of AZC did not affect ALP expression. (C) Culture of hMSCs in the presence of AZC for 21 days resulted in a significant decrease in calcium accumulation $*p < 0.05$. (D) Collagen deposition by hMSCs cultured for 21 days in the presence of AZC was partially reduced. AZC, L-2-azetidine carboxylic acid.

example, MC3T3-E1 cells, a murine preosteoblastic cell line, did not differentiate into the osteogenic lineage in the absence of ascorbic acid.⁴² These observations are in line with our data showing that in the absence of ascorbic acid, hMSCs produce minimal amounts of collagen, leading to an inhibition of proliferation. In most tissue engineering applications, cell number is of critical importance, and it is therefore recommended to expand cells in the presence of ascorbic acid because the yield at first passage is higher in its presence. The mitogenic effect observed in the presence of ascorbic acid can be ascribed either to the role of ECM as a growth factor reservoir or direct activation of a mitogenic pathway by ascorbic acid. In the former, the growth factors secreted by the cells are soluble in the medium in the absence of collagen, and lost during medium refreshment. In the latter case, integrin signaling may be impaired and the downstream signals that lead to activation of mitogen-activated protein kinase activation are absent in the absence of collagen.^{43,44} In addition to the mitogenic role of ECM we also observed an effect on osteogenesis. We observed that ALP expression and calcium accumulation were both decreased in the absence of ascorbic acid, pointing toward a role of collagen in osteogenic differentiation.¹⁸ Interestingly, the effects on ALP expression and calcium accumulation were similar irrespective of the initial presence of ascorbic acid and further culture without ascorbic acid for the remaining period of the assay. In that case, the effect of ECM deposited when cells were exposed to ascorbic acid most likely disappeared upon removal because ECM was remodeled and degraded by matrix-degrading enzymes without new deposition due to the absence of ascorbic acid. In this respect, it is interesting to observe that cathepsin K, an osteoclast-related gene also expressed in osteoblasts, is one of the genes highly expressed in the presence of ascorbic acid, indicating that concomitant with ECM deposition there is a signal to remodel it, which may lead to release of growth factors from ECM.⁴⁵ Interestingly, in the presence of ascorbic acid, several interferon-induced proteins were upregulated. Recently, it has been shown that interferon-gamma is a strong inhibitor of osteoclast activity and, further, its exogenous addition accelerates osteogenic differentiation of hMSCs.⁴⁶ This poses a possibility that collagen represents an anti-osteoclastic and pro-osteogenic signal by activating interferon-mediated signaling.

Further mining of the microarray data suggested that in the absence of ascorbic acid, differentiation into other lineages was favored since genes involved in differentiation toward those lineages were upregulated. This would indicate that ascorbic acid not only has an effect on proliferation of the cells but also plays a role in cell fate decision by controlling the switch between different lineages. Despite the positive effect of collagen on osteogenesis, our gene expression data suggest that in its presence the multilineage potential of hMSCs is maintained, which is similar to the effect of dex. Despite the fact that dex is generally used as an osteogenic factor *in vitro*, expansion of hMSCs in its presence does not impair multilineage differentiation. To favor osteogenic differentiation, not only dex is necessary but also the presence of an ECM that serves as a template for mineralization and as a reservoir of growth factors. Likewise, expansion of hMSCs in the absence of ascorbic acid did not hamper the osteogenic potential as seen by calcium accumulation.

Conclusion

We have shown that collagen plays a positive role in proliferation and osteogenic differentiation of hMSCs, and that addition of ascorbic acid to the culture medium favors *in vivo* bone formation by hMSCs. Additionally, our genome-wide expression data show that both in the presence and in the absence of collagen, hMSCs keep their multipotency, and we have disclosed an interesting cross-talk between collagen and matrix remodeling.

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Disclosure Statement

No competing financial interests exist.

References

1. Bruder, S.P., and Fox, B.S. Tissue engineering of bone. Cell based strategies. Clin Orthop Relat Res (367 Suppl) S68, 1999.
2. Bruder, S.P., Kurth, A.A., Shea, M., Hayes, W.C., Jaiswal, N., and Kadiyala, S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res 16, 155, 1998.
3. Barry, F.P., and Murphy, J.M. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 36, 568, 2004.
4. Macchiarini, P., Jungebluth, P., Go, T., Asnaghi, M.A., Rees, L.E., Cogan, T.A., Dodson, A., Martorell, J., Bellini, S., Parnigotto, P.P., Dickinson, S.C., Hollander, A.P., Manterd, S., Conconi, M.T., and Birchall, A. Clinical transplantation of a tissue-engineered airway. Lancet 372, 2023, 2008.
5. Atala, A., Bauer, S.B., Soker, S., Yoo, J.J., and Retik, A.B. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 367, 1241, 2006.
6. Ahlmann, E., Patzakis, M., Roidis, N., Shepherd, L., and Holtom, P. Comparison of anterior and posterior iliac crest bone grafts in terms of harvest-site morbidity and functional outcomes. J Bone Joint Surg 84A, 716, 2002.
7. Arrington, E.D., Smith, W.J., Chambers, H.G., Bucknell, A.L., and Davino, N.A. Complications of iliac crest bone graft harvesting. Clin Orthop Relat Res 329, 300, 1996.
8. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science 284, 143, 1999.
9. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R.J., Keating, A., Prockop, D.J., and Horwitz, E.M. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315, 2006.
10. Caplan, A.I. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng 11, 1198, 2005.
11. Yoshida, K., Chambers, I., Nichols, J., Smith, A., Saito, M., Yasukawa, K., Shoyab, M., Taga, T., and Kishimoto, T. Maintenance of the pluripotential phenotype of embryonic

- stem cells through direct activation of gp130 signalling pathways. *Mech Dev* **45**, 163, 1994.
12. Solchaga, L.A., Penick, K., Porter, J.D., Goldberg, V.M., Caplan, A.I., and Welter, J.F. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* **203**, 398, 2005.
13. Siddappa, R., Fernandes, H., Liu, J., van Blitterswijk, C., and de Boer, J. The response of human mesenchymal stem cells to osteogenic signals and its impact on bone tissue engineering. *Curr Stem Cell Res Ther* **2**, 209, 2007.
14. Siddappa, R., Martens, A., Doorn, J., Leusink, A., Olivo, C., Licht, R., van Rijn, L., Gaspar, C., Fodde, R., Janssen, F., van Blitterswijk, C., and De Boer, J. cAMP/PKA pathway activation in human mesenchymal stem cells *in vitro* results in robust bone formation *in vivo*. *Proc Natl Acad Sci U S A* **105**, 7281, 2008.
15. de Boer, J., Licht, R., Bongers, M., van der Klundert, T., Arends, R., and van Blitterswijk, C. Inhibition of histone acetylation as a tool in bone tissue engineering. *Tissue Eng* **12**, 2927, 2006.
16. Bi, Y., Ehrichtou, D., Kilts, T.M., Inkson, C.A., Embree, M.C., Sonoyama, W., Li, L., Leet, A., Seo, B., Zhang, L., Shi, S., and Young, M. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* **13**, 1219, 2007.
17. Fashena, S.J., and Thomas, S.M. Signalling by adhesion receptors. *Nat Cell Biol* **2**, E225, 2000.
18. Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S.P. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J Cell Biochem* **64**, 295, 1997.
19. Kao, W.W., Flaks, J.G., and Prockop, D.J. Primary and secondary effects of ascorbate on procollagen synthesis and protein synthesis by primary cultures of tendon fibroblasts. *Arch Biochem Biophys* **173**, 638, 1976.
20. Geesin, J.C., Hendricks, L.J., Gordon, J.S., and Berg, R.A. Modulation of collagen synthesis by growth factors: the role of ascorbate-stimulated lipid peroxidation. *Arch Biochem Biophys* **289**, 6, 1991.
21. Lin, T.M., Tsai, J.L., Lin, S.D., Lai, C.S., and Chang, C.C. Accelerated growth and prolonged lifespan of adipose tissue-derived human mesenchymal stem cells in a medium using reduced calcium and antioxidants. *Stem Cells Dev* **14**, 92, 2005.
22. Choi, K.-M., Seo, Y.-K., Yoon, H.-H., Song, K.-Y., Kwon, S.-Y., Lee, H.-S., and Park, J.K. Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. *J Biosci Bioeng* **105**, 586, 2008.
23. Sudo, H., Kodama, H.A., Amagai, Y., Yamamoto, S., and Kasai, S. *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* **96**, 191, 1983.
24. Torii, Y., Hitomi, K., and Tsukagoshi, N. L-ascorbic acid 2-phosphate promotes osteoblastic differentiation of MC3T3-E1 mediated by accumulation of type I collagen. *J Nutr Sci Vitaminol (Tokyo)* **40**, 229, 1994.
25. Franceschi, R.T., and Iyer, B.S. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res* **7**, 235, 1992.
26. Chang, Y., Ueng, S.W., Lin-Chao, S., and Chao, C.C. Involvement of Gas7 along the ERK1/2 MAP kinase and SOX9 pathway in chondrogenesis of human marrow-derived mesenchymal stem cells. *Osteoarthritis Cartilage* **16**, 1403, 2008.
27. Zuckerman, K.S., Rhodes, R.K., Goodrum, D.D., Patel, V.R., Sparks, B., Wells, J., Wicha, M.S., and Mayo, L.A. Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long-term murine bone marrow cultures. *J Clin Invest* **75**, 970, 1985.
28. Gordon, M.Y. Extracellular matrix of the marrow microenvironment. *Br J Haematol* **70**, 1, 1988.
29. Moore, K.A., and Lemischka, I.R. Stem cells and their niches. *Science* **311**, 1880, 2006.
30. Fernandes, H., Dechering, K., Van Someren, E., Steeghs, I., Apotheker, M., Leusink, A., Bank, R., Janeczke, K., van Blitterswijk, C., and De Boer, J. The role of collagen cross-linking in differentiation of human mesenchymal stem cells and MC3T3-E1 cells. *Tissue Eng Part A* **15**, 3857, 2009.
31. Mauney, J.R., Kaplan, D.L., and Volloch, V. Matrix-mediated retention of osteogenic differentiation potential by human adult bone marrow stromal cells during *ex vivo* expansion. *Biomaterials* **25**, 3233, 2004.
32. Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677, 2006.
33. Ott, H.C., Matthiesen, T.S., Goh, S.K., Black, L.D., Kren, S.M., Netoff, T.I., and Taylor, D.A. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med* **14**, 213, 2008.
34. Chen, X.-D., Dusevich, V., Feng, J.Q., Manolagas, S.C., and Jilka, R.L. Extracellular matrix made by bone marrow cells facilitates expansion of marrow-derived mesenchymal progenitor cells and prevents their differentiation into osteoblasts. *J Bone Miner Res* **22**, 1943, 2007.
35. Postovit, L.-M., Seftor, E.A., Seftor, R.E.B., and Hendrix, M.J.C. A Three-dimensional model to study the epigenetic effects induced by the microenvironment of human embryonic stem cells. *Stem Cells* **24**, 501, 2006.
36. de Bruijn, J.D., van den Brink, I., Mendes, S., Dekker, R., Bovell, Y.P., and van Blitterswijk, C.A. Bone induction by implants coated with cultured osteogenic bone marrow cells. *Adv Dent Res* **13**, 74, 1999.
37. Bank, R.A., Jansen, E.J., Beekman, B., and te Koppele, J.M. Amino acid analysis by reverse-phase high-performance liquid chromatography: improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate. *Anal Biochem* **240**, 167, 1996.
38. Bank, R.A., Beekman, B., Verzijl, N., de Roos, J.A., Sakkee, A.N., and TeKoppele, J.M. Sensitive fluorimetric quantitation of pyridinium and pentosidine crosslinks in biological samples in a single high-performance liquid chromatographic run. *J Chromatogr B Biomed Sci Appl* **703**, 37, 1997.
39. Dennis, G., Sherman, B., Hosack, D., Yang, J., Gao, W., Lane, H.C., and Lempicki, R. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* **4**, P3, 2003.
40. Aubin, J.E. Advances in the osteoblast lineage. *Biochem Cell Biol* **76**, 899, 1998.
41. Prockop, D.J., and Kivirikko, K.I. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* **64**, 403, 1995.
42. Xiao, G., Cui, Y., Ducy, P., Karsenty, G., and Franceschi, R.T. Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol Endocrinol* **11**, 1103, 1997.

43. Adams, J.C., and Watt, F.M. Regulation of development and differentiation by the extracellular matrix. *Development* **117**, 1183, 1993.
44. Pozzi, A., Wary, K.K., Giancotti, F.G., and Gardner, H.A. Integrin alpha 1beta 1 mediates a unique collagen-dependent proliferation pathway *in vivo*. *J Cell Biol* **142**, 587, 1998.
45. Mandelin, J., Hukkanen, M., Li, T.-F., Korhonen, M., Liljestrom, M., Sillat, T., Hanemaaijer, R., Salo, J., Santavirta, S., and Konttinen, Y. Human osteoblasts produce cathepsin K. *Bone* **38**, 769, 2006.
46. Duque, G., Huang, D.C., Macoritto, M., Rivas, D., Yang, X.F., Ste-Marie, L.G., and Krener, R. Autocrine regulation of interferon {gamma} in mesenchymal stem cells plays a role in early osteoblastogenesis. *Stem Cells* **27**, 550, 2009.

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