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Collagen type I hydrogel allows migration, proliferation and osteogenic differentiation of rat bone marrow stromal cells

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Abstract

Hydrogels are potentially useful for many purposes in regenerative medicine including drug and growth factor delivery, as single scaffold for bone repair or as a filler of pores of another biomaterial in which host mesenchymal progenitor cells can migrate in and differentiate into matrix-producing osteoblasts. Collagen type I is of special interest as it is a very important and abundant natural matrix component. The purpose of this study was to investigate whether rat bone marrow stromal cells (rBMSCs) are able to adhere to, to survive, to proliferate and to migrate in collagen type I hydrogels and whether they can adopt an osteoblastic fate. rBMSCs were obtained from rat femora and plated on collagen type I hydrogels. Prior to harvest by day 7, 14, and 21, hydrogels were fluorescently labeled, cryo-cut and analyzed by fluorescent-based and laser scanning confocal microscopy to determine cell proliferation, migration, and viability. Osteogenic differentiation was determined by alkaline phosphatase activity. Collagen type I hydrogels allowed the attachment of rBMSCs to the hydrogel, their proliferation, and migration towards the inner part of the gel. rBMSCs started to differentiate into osteoblasts as determined by an increase in alkaline phosphatase activity after two weeks in culture. This study therefore suggests that collagen type I hydrogels could be useful for musculoskeletal regenerative therapies.

Keywords

Collagen type I hydrogel; bone marrow stromal cells; cell migration; osteogenic differentiation; bone regeneration

INTRODUCTION

In musculoskeletal medicine an increasing clinical need exists for bone regeneration and grafting strategies to treat bony voids after spinal fusion, maxillofacial surgery, implant fixation, resection of primary tumors and metastases, or trauma.¹ Autologous bone is commonly used for grafting as it contains many crucial features such as mesenchymal

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progenitor cells, osteoinductive factors, and an osteoconductive matrix that together support skeletal healing. However, its use is restricted by limited graft availability and donor site morbidity, two major disadvantages that ultimately drive the quest for alternative approaches to efficiently fill any kind of bony defect.²

A critical component for the successful repair of osseous defects are bone marrow stromal cells (BMSCs).^{3,4} BMSCs retain the ability to differentiate along different lineages and can become chondroblasts, adipocytes, fibroblasts, or osteoblasts,⁵ a feature that makes BMSCs the premier cellular source for regenerative approaches in the musculoskeletal field. Recently it has been proposed that the main function of transplanted BMSCs is not to directly conduct bone formation but rather to contribute to a regenerative environment by which host osteoprogenitors can be recruited to the site of repair and differentiate into matrix-producing osteoblasts.^{6,7}

A promising concept in regenerative medicine of the musculoskeletal system is the use of biological grafts as tissue substitutes. In order to fulfill clinical requirements, these materials ideally provide a minimal primary stability to the site of repair, are biocompatible, and preferentially support local bone healing.⁸

Hydrogels are biodegradable synthetic or natural polymers that can be manufactured in large quantities and whose mechanical and biological properties can be varied over a wide range, thereby opening novel treatment opportunities. Hydrogels can be used for drug or growth factor delivery, the grafting of irregular shaped defects, or to fill the pores of a three-dimensional interconnected network that can sufficiently stabilize the site of skeletal repair.⁹ Hydrogels may mimic many aspects of the natural extracellular environment, and modulate cell function by allowing the diffusion of nutrients, metabolites, and growth factors.¹⁰ Many natural and synthetic hydrogels have been investigated for their potential use in regenerative medicine such as agarose,¹¹ alginate,¹² chitosan,^{13,14} fibrin,¹⁵ silk,^{16,17} hyaluronic acid,¹⁸ cyclic acetal,¹⁹ oligo (poly-(ethylene glycol) fumarate) (OPF)^{20,21} and collagen.²² Natural hydrogels have structural similarity with the physiological extracellular matrix and are considered biocompatible. Collagen is the most widely used tissue-derived natural polymer, and it is the prevailing component of the extracellular matrix of many tissues including skin, ligament, cartilage, tendon, and bone. Collagen is composed of specific combinations of amino acid sequences that are recognized by cells thereby facilitating cell attachment and migration and it is subject for degradation by enzymes secreted from these cells (i.e., collagenase). Collagen is frequently used as a tissue culture matrix due to its ability to induce attachment of many different cell types and its cell-based degradation. Clinically, collagen gels have been applied for the reconstruction of whole organs such as skin, blood vessels, and small intestine.¹⁰

If collagen type I hydrogels are used as a biomaterial for bone regeneration, it is highly desirable that host BMSCs attach to and migrate into the hydrogel, where they keep proliferating. In addition, BMSCs are expected to initiate commitment to the osteoblast lineage to ultimately become a fully functional matrix-producing osteoblast. We therefore investigated the migration, proliferation, and osteogenic differentiation of rat bone marrow stromal cells (rBMSCs) in collagen type I hydrogels.

MATERIALS AND METHODS

Bone marrow stromal cell isolation and culture

For the use of animals the NIH guidelines for the care and use of laboratory animals have been followed. Rat bone marrow stromal cells (rBMSCs) were harvested from both femora of four 8 weeks old male Sprague-Dawley rats. Briefly, rats were euthanized using 4%

isofluorane in CO₂. Both femora were aseptically excised and surrounding soft tissue was removed. Both ends of the femora were clipped, and the bone marrow was flushed from the shaft into DMEM/F-12 cell culture medium supplemented with 10% fetal bovine serum (FBS), fungizone 20 µg/ml and gentamicin sulfate 20 µg/ml (all Life Technologies Inc., Grand Island, NY) using a 5 ml syringe and a 18-gauge needle (both Becton Dickinson & Co, Franklin Lakes, NJ). The antibiotic composition differed from that used in other cell culture procedures described here and was chosen to avoid contamination during the cell harvest. Subsequently, rBMSCs were centrifuged and the cell pellets were re-suspended in DMEM/F-12 medium containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (all Life Technologies Inc., Grand Island, NY). Cells derived from different animals were combined and grown in 75 cm² cell culture flasks (Becton Dickinson & Co, Franklin Lakes, NJ) at 37°C and 5% CO₂ in a humidified atmosphere. Before the cell layer became confluent, cells were detached with trypsin (Life Technologies, Grand Island, NY) and cultured for 2 additional passages. The medium was changed every second day. To induce osteogenic differentiation, 100 µg/ml L-ascorbic acid phosphate (Wako Pure Chemical Industries LTD, RI), 10 mM β-glycerophosphate, and 2.55 mM dexamethasone (both Sigma-Aldrich Corp., St. Louis, MO) were supplemented to the medium.

Collagen type I hydrogel preparation

Purified bovine dermal collagen type I (Vitrogen®, Cohesion, Palo Alto, CA) was used as scaffold material. The hydrogel was formed by collagen fibril self-assembly inside of a trans-well insert (12 mm diameter, 4 µm membrane pore size) (Corning Inc, Corning, NY). Briefly, the pH of the collagen type I solution was adjusted to 7.4 according to the manufacturer's protocol. Next, liquid collagen type I was transferred to a trans-well insert and filled up to 2/3 of the insert. Collagen type I fibril self-assembly was achieved by subsequent incubation at 37°C in normal atmosphere for 60 minutes. Collagen type I gels were sterilized over night using anprolene sterilizing gas ampoules (Anderson Products, Inc., Haw River, NC). After rinsing the collagen type I hydrogels two times with 1x phosphate-buffered saline (PBS), the hydrogels were conditioned for 12–24 h using cell culture medium.

Preparation of cell-seeded collagen type I hydrogels and sample processing

Conditioned collagen type I hydrogels and control tissue culture plates were seeded with 5×10^4 rBMSCs/cm². Collagen type I constructs were harvested after 7, 14, and 21 days. Cells from control cultures were harvested and count by day 7 and by day 14. For each time point, 9 hydrogels were seeded with rBMSCs and all assays were performed in triplicate. For subsequent DNA quantification and measurement of alkaline phosphatase activity, hydrogels were immediately frozen at –80°C. For cryosectioning and subsequent histomorphometric analysis, hydrogels were embedded in Tissue-Tek® O.C.T. Compound 4583 (Sakura Finetek, Torrance, CA) and stored at –80°C until analysis. Laser scanning confocal microscopy was performed using fresh and hydrated gels immediately after labeling with the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) as recommended by the manufacturer.

Histological sections

Frozen hydrogels were embedded In Tissue-Tek® O.C.T. Compound 4583 and cut into 6 µm sagittal sections at –20°C using a cryostat (Leica Jung CM 3000, Wetzlar, Germany). Sections were taken from the central portion of three different hydrogels at each time point and air dried at room temperature on a microscope slides (Fisher Scientific, Pittsburgh, PA). Samples were labeled using the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Inc., Eugene, OR), air dried, overlaid with mounting medium, and covered with a cover slip.

All samples were protected from light and kept at room temperature until fluorescent microscopy was performed.

Fluorescent microscopy

For each analytic time point, 6 labeled cryo-sections derived from the center of three different hydrogels (18 sections in total) were visualized using a fluorescent microscope (Olympus BH2-RFCA, Olympus, Melville, NY). The number of cells that remained on the hydrogel surface and the number of cells that migrated into the hydrogel was determined as well as the distance between each migrated cell and the hydrogel surface. Each sample was analyzed in three rows with six fields per row using the Osteomeasure Bone Histomorphometry System 4.0 (Osteometrics Inc., Atlanta, GA).

Confocal microscopy

Three calcein AM/Ethidium homodimer-1-stained hydrogel/cell- constructs per time point were placed on a glass slide (Fisher Scientific, Pittsburgh, PA), and analyzed using a LSM 510 confocal laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY). Calcein AM was excited at 488 nm by an argon/krypton laser. Emission was selected with a 505–550 nm bandpass filter. Ethidium homodimer-1 was excited at 568 nm by the argon/krypton laser. Emission was selected with a 585 nm longpass filter. Images were collected at 512×512 pixel resolution using a C-Apochromat 10x/0.45 water immersion lens. The software used was part of the LSM 510 system.

Alkaline phosphatase assay

In order to determine the commitment of the mesenchymal precursor cells to the osteoblast lineage, alkaline phosphatase (ALP) activity was assayed (Sigma Chemical Co., St. Louis, MO) in 3 samples in triplicate. Briefly, cell culture medium was removed and cell-seeded collagen type I hydrogels were washed twice with 1 × PBS and kept at –80°C until analysis. Cell-seeded hydrogels were thawed and gently homogenized in the trans-well insert using 0.3 ml AP-substrate/AP-Buffer mix (1:1) followed by an incubation at 37°C for 30 minutes. The reaction was stopped after adding 0.3 ml NaOH (50 mM). Aliquots (200 µl) of the samples were transferred to a 96-well plate (Corning Inc, Corning, NY). A standard calibration curve was established using a serial dilution of p-nitrophenol according to the manufacturer's protocol. Emission was detected at 562 nm using a plate reader (Spectra Max Plus, Molecular Devices, Sunnyvale, CA) followed by the analysis of the excitation at 645 nm. Quantifications were done at least in duplicate. All data were normalized to the total amount of DNA.

DNA quantification

To normalize the ALP activity, dsDNA derived from the cell-containing collagen type I hydrogels was quantified using the cyanine dye PicoGreen® according to the manufacturer's recommendations (Molecular Probes, Inc., Eugene, OR). After excitation at 480 nm fluorescence emission intensity was detected at 520 nm using the plate reader. Samples were analyzed in triplicate.

Statistical Analysis

All results were expressed as the mean ± SEM. Statistical analysis was performed by ANOVA-test with Fisher's PLSD and paired t-test. P-values < 0.05 were considered statistically significant.

RESULTS

Migration, viability and proliferation of rBMSCs

rBMSCs located both on the surface and inside of collagen type I hydrogels were fluorescently labeled using the Live/Dead[®] Viability/Cytotoxicity Kit and analyzed microscopically after one, two, and three weeks of osteogenic culture. The hydrogels were intact during the entire experimental period without any decrease in stability. Fluorescent microscopy (Fig. 1A–C) revealed a progressive migration of rBMSCs into collagen type I hydrogels scaffold during the time of investigation. By day 7, a fraction of rBMSCs had started to migrate towards the center of the hydrogel matrix while the majority of the seeded rBMSCs remained densely packed on the hydrogel surface (Fig. 1A–C). The cells that had started to migrate after one week of culture, continued to migrate in an undulated or cone-like pattern for the remaining 14 days of the experiment (Fig. 1B+C).

In the next step we quantified the distance of migration during the three weeks of investigation. By day 7, the cells located underneath the hydrogel surface had migrated an average distance of $25 \pm 2 \mu\text{m}$, which significantly increased to $35 \pm 2 \mu\text{m}$ ($p < 0.05$) by day 14 and further increased to $47 \pm 2 \mu\text{m}$ ($p < 0.05$) after three weeks of culture under osteogenic conditions (Fig. 2). Almost all of the rBMSCs on the hydrogel surface and also inside the hydrogel were viable and highly interconnected as qualitatively determined by laser-scanning confocal microscopy (Fig. 3A+B).

Next we quantified the total number of all rBMSCs using histomorphometric analysis of fluorescently labeled frozen hydrogel sections. In addition, we individually determined the number of cells that migrated into the hydrogel as well as the number of cells that remained on the surface. As depicted by the full-length of the combined bars in figure 4A (black & white parts together), the total number rBMSCs on the hydrogel surface and inside the gel increased progressively. Briefly, by day 7 a total number of 523 ± 51 rBMSCs were counted per standardized area of analysis (Fig. 4A). During the experimental course the total number of cells significantly increased to 968 ± 53 cells/section ($p < 0.05$) by day 14 and continued to increase to 1119 ± 30 cells/section ($p < 0.05$) by day 21 (Fig. 4A), suggesting a positive net proliferation of rBMSCs. To further analyze the influence of the collagen type I hydrogel on rBMSCs proliferation within the different zones of the hydrogel, we separately quantified the number of rBMSCs on the hydrogel surface and of the cells that resided inside the gel. After one week of culture, the number of rBMSCs that remained on the surface was 363 ± 45 while 161 ± 19 cells had been migrated into the hydrogel. We observed a significant increase of the number of rBMSCs at both sites during the following two weeks of incubation. Briefly, by day 14, 607 ± 35 rBMSCs were counted on the surface while 361 ± 28 cells ($p < 0.05$) were found inside the gel. These numbers further increased to 732 ± 41 cells ($p < 0.05$) on the surface and to 388 ± 11 migrated rBMSCs ($p < 0.05$) by day 21 (Fig. 4A).

Next we investigated the effect of the collagen type I hydrogel on cell proliferation. Briefly, the same number of rBMSCs per area was plated on hydrogels and on control tissue culture plates and the increase in cell number in both systems was compared. Between day 7 and day 14, the number of cells grown in control tissue culture plates increased 1.39 ± 0.04 -fold, while the cells that had contact with the hydrogels proliferated 1.71 ± 0.05 -fold ($p < 0.05$) (Fig. 4B).

Although the total number of all cells and the number of both individual cell fractions inside and outside the hydrogel increased linearly over time in osteogenic culture, the fraction of rBMSCs inside the hydrogel remained the same during the entire experiment and accounted for about one third (day 7: $31\% \pm 3\%$; day 14: $37\% \pm 1.8\%$; day 21: $35\% \pm 1.9\%$ rBMSCs,

$p > 0.05$ between time points) of the entire cell population (Fig. 4C). These results indicate that collagen type I hydrogels allow mesenchymal precursor cells to proliferate both on top of the hydrogel and inside the gel matrix, suggesting that cell-intrinsic properties may determine whether a cell starts to migrate or not.

Osteogenic differentiation of rBMSCs

In addition to proliferation, survival and migration, we studied the ability of our experimental system to allow rBMSCs to differentiate along the osteoblast lineage, as it would be highly desirable for collagen type I hydrogels if used in musculoskeletal regeneration.

Alkaline phosphatase (ALP) is a cell membrane-associated phosphatase that is involved in the onset of extracellular matrix mineralization and considered as a relatively early marker in the cascade of osteoblast differentiation.²³ We quantified ALP activity of rBMSCs in collagen type I hydrogels and normalized the values to the total amount of DNA. This assay was then used as a read-out for the early osteoblastic differentiation of mesenchymal progenitor cells.

Our results show that by day 7 the normalized alkaline phosphatase activity found in rBMSC-seeded hydrogels was 11.9 ± 0.7 (AP [μ M]/DNA [ng]) and remained unchanged until day 14 (10.4 ± 1.3 (AP [μ M]/DNA [ng])). During the third week of culture under osteogenic conditions, alkaline phosphatase activity started to rise reaching a significantly increased activity of 16.2 ± 1.3 (AP [μ M]/DNA [ng]) by day 21, thereby demonstrating the differentiation of rBMSCs along the osteoblast lineage (Fig. 5).

Collectively, these data indicate that rBMSCs remained functional inside the collagen type I hydrogels because they underwent cell division, they migrated into the hydrogel, and because they started to differentiate into osteoblasts.

DISCUSSION

This study was aimed at investigating the ability of primary rat bone marrow stromal cells (rBMSCs) to attach to, to migrate into, and to proliferate inside collagen type I hydrogels and to eventually adopt an osteoblastic fate during three weeks of culture. Our results demonstrate that collagen type I hydrogels allow the attachment and proliferation of anchorage-dependent rBMSCs. Almost all of the cells remained viable and about one third of the cell pool begun to migrate into the hydrogel in a time-dependent fashion. Finally, we have shown that osteoblast differentiation of the mesenchymal precursor cells was successfully initiated between two and three weeks of culture. These data suggest that collagen type I hydrogels might be suitable for novel strategies in musculoskeletal regeneration.

Bony defects are often a consequence of an infection, a congenital deformity, a tumor, but are most often the result of a fracture. Autologous bone is currently the material of choice for grafting but it is limited in quantity and its harvest is often associated with donor site morbidity.^{24–26} Thus, alternative approaches to fill bony voids are needed in addition to autografting.

Bone marrow harbors stromal cells that function as multipotent progenitor cells and possess a strong bone-forming capacity *in vivo*.^{27–29} Current strategies in regenerative medicine are largely based on the use of *ex vivo* expanded progenitor cells, which can be combined with suitable biomaterials prior to implantation in skeletal lesions.^{5,30,31} However, more recent models propose that mainly host progenitor cells build new bone after they have been

recruited to the site of repair to which transplanted cells are contributing to.^{6,7} Based on this concept, it is critical that the recruited host cells can enter and reside inside the graft to ultimately form new bone where it is needed.

Collagen type I hydrogels are natural polymers that have been proven for a long time to be useful for many clinical applications.^{10,22,32} Because collagen type I has been shown in several studies to provide appropriate extracellular cues to support physiological cell functions³¹, we hypothesized that it may also allow the attachment, proliferation, migration, and osteoblastic differentiation of anchorage-dependent bone marrow stromal cells. We therefore addressed these questions using a model system in which rBMSCs were cultured for three weeks under osteogenic conditions on the surfaces of collagen type I hydrogels.

Here we provide experimental evidence that the vast majority of precursor cells remained viable both on the surface and inside the hydrogel, suggesting that nutrients and oxygen were constantly accessible inside the aqueous hydrogel. This extracellular environment also allowed the formation of intercellular connections inside the hydrogel, which is considered as a prerequisite for the successful proliferation of cells inside polymeric materials.³³ In our study collagen type I hydrogels allowed the attachment of anchorage-dependent rBMSCs and promoted cell proliferation compared to control cultures. About one third of the entire cell population separated themselves from the hydrogel surface and started to actively migrate towards the opposite site of the hydrogel. The undulated or cone-like penetration pattern could be the consequence of a heterogeneous fibril assembly, which could have led to the formation of pathways by which oxygen and nutrients may have unevenly reached those cells. A recently published study also demonstrated an irregular accumulation of osteoblastic differentiated rat mesenchymal cells in synthetic hydrogels and concluded that the polymer network structure encouraged heterogeneous nucleation of calcium phosphate crystals.²⁰ This group and other groups demonstrated that hydrogel properties positively influence osteogenic differentiation of mesenchymal progenitor cells.^{20,31}

We obtained primary cells after flushing rat femora, a method that yields a precursor cell pool that is heterogeneous in terms of lineage pre-commitment and differentiation status.³⁴ Some cells of this heterogeneous population were incapable to enter the collagen type I matrix, which could have been the result of a differentially expressed or activated integrin receptor system.^{35,36} Differences in integrin signaling might then have led to a differentially activated transcriptional machinery or a somehow altered cytoskeletal engagement with subsequent differences in the migratory phenotype of these cells.³⁶⁻³⁹ Cells that migrated demonstrated an increase in the average migration distance and reached about 50 μm in three weeks, which equals about 10 cell diameters in our system.

Another important aspect analyzed in this study is osteogenic differentiation of the precursor cells. Alkaline phosphatase (ALP) is a marker of osteoblast differentiation and its activity normally rises after several days in culture and peaks after one to two weeks.²³ In our system the ALP activity started to increase after day 14 and reached a significantly elevated level by day 21. The increase in ALP activity seen here is modestly delayed compared to other studies reporting 2D cell culture conditions and could be due to the process of cell attachment to the hydrogel, initiation of cell proliferation and/or cell migration into the collagen type I hydrogel matrix. Apparently these cellular programs needed to be at least initiated or even completed before a coordinated osteoblast differentiation program could start.²³ This is consistent with the observation that the upregulation of ALP mRNA is linked with the termination of the proliferation stage.^{23,40} In addition, ALP is a membrane-bound molecule, a circumstance that could have complicated its extraction from cells that were surrounded by the hydrogel thereby limiting the possibility to detect early trends in its upregulation. However, these data are consistent with a study in which osteogenic

differentiation of stromal cells was detected after 21 days of culture in thermally cross-linked OPF hydrogels.^{20,41}

CONCLUSION

We addressed the questions whether primary rBMSCs can attach to and migrate into collagen type I hydrogels and if these cells can proliferate and differentiate along the osteoblastic lineage. Our results indicate that collagen type I hydrogels indeed allow the successful initiation of all these cellular processes. A subset of cells started to migrate into the matrix while almost all cells remained viable and proliferating while expressing a marker of the osteoblast lineage. These data suggest that *in vivo* collagen type I is likely to allow host rBMSCs to attach to and to enter the hydrogel and to undergo osteoblast differentiation. Our study therefore contributes to the development of novel bone grafts for the future use in regenerative medicine.

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Figure 1.

Qualitative analysis of migration, and proliferation of rBMSCs in collagen type I hydrogels during a three weeks period. Collagen type I hydrogels were seeded with rBMSCs. Hydrogels (n=3) were labeled with Calcein AM/Ethidium homodimer-1 prior to analysis by day 7 (A), day 14 (B), and day 21 (C). For fluorescent microscopy, cell-matrix constructs were immediately frozen and cryo-cut in sagittal sections. Cells are shown in red. The image demonstrates the increase of the overall cell number and the progressive increase in the migration distance of some rBMSCs in collagen type I hydrogels. The cell migration front is highlighted with a white dotted line. Representative images were taken at 100x magnification.

Figure 2.

Quantitative analysis of the distance of rBMSC migration in collagen type I hydrogels during three weeks of culture. Collagen type I hydrogels were overlaid with rBMSCs and cultured for 21 days. Prior to analysis, samples were labeled with a fluorescent dye and cryo-cut. The distance between each cell and the hydrogel surface was determined by standardized histomorphometric analysis. Shown are the migration distances after 7 days, 14 days, and 21 days. Data represent means $\pm$ standard deviation for three samples ($n=3$, 6 sections/sample). *Indicates statistical significance compared to day 7 ($p<0.05$), and # indicates statistical significance compared to day 14 ($p<0.05$).

Figure 3.

Viability of rBMSCs in collagen type I hydrogels. Collagen type I hydrogels (n=3) were seeded with rBMSCs and incubated for 21 days. Prior optical analysis by laser scanning confocal microscopy, native cell-matrix constructs were labeled with Calcein AM/Ethidium homodimer-1. Shown are representative images taken by day 21 in two different magnifications (A=100x, B=400x). Viable cells appear in green.

Figure 4A

Figure 4B

Figure 4C

Figure 4.

Proliferation of rBMSCs and relation of migrated to non-migrated rBMSCs after plating on collagen type I hydrogels. Cell-seeded hydrogels were cultured for a period of 21 days. Samples were fluorescently labeled and cryo-sectioned by day 7, day 14, and day 21. The total number of all rBMSCs and the number of migrated cells as well as the number of cells that remained on the gel surface was individually determined by standardized histomorphometric analysis. The graph shows a time-related increase of the total and the subgroup-related cell number. Data represent means $\pm$ standard deviation for three samples ($n=3$, 6 sections/sample). *Indicates statistical significance compared to day 7 ($p<0.05$), and # indicates statistical significance compared to day 14 ($p<0.05$) (A). A proliferation factor was calculated based on the increase in cell number between day 7 and day 14. Shown are the proliferation factors for cells grown on hydrogels and for cells grown in control culture (B). The percentage of the migrated cells is about one third of all rBMSCs and remains unchanged during the experiment ($p>0.05$) (C).

Figure 5.

Osteoblast differentiation of rBMSCs in collagen type I hydrogels during three weeks of osteogenic culture. Collagen type I hydrogels were seeded with rBMSCs and incubated for a period of 21 days in medium containing osteogenic supplements. Hydrogels were homogenized after 7 days, 14 days, and 21 days. Alkaline phosphatase activity was quantified and normalized to DNA. Osteogenic differentiation of rBMSCs started after two weeks of culture and led to a significantly increased level of ALP activity by day 21. Data represent means $\pm$ standard deviation for $n=3$. # Indicates statistical significance compared to day 14 ($p<0.05$).