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Collagen—Dexamethasone and Collagen-D_3 Scaffolds for Bone Tissue Engineering

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The aim of the study was to test the biocompatibility and osteoinductive potential of collagen with dexamethasone (Dex) and collagen with 1, 25 dihydroxycholecalciferol (D_3) scaffolds on two human osteoblast cell lines, MG63 and hFOB1.19. The cell morphology was examined by SEM, the scaffolds colonization was monitored by fluorescence microscopy, viability by MTT assay and cells differentiation by PCR. The two utilized scaffolds sustained biointegration/proliferation of human osteoblast cells. However, collagen-Dex and collagen-D_3 scaffolds promoted osteogenic activity of MG63 cells incresing the gene expression of osteonectine, osteocalcin and BSP II whereas no osteoinductive effect on hFOB1.19 cells was detected.

Keywords Biocompatibility; human osteoblast cells; osteoinduction; 3D scaffolds

Introduction

The application of scaffolds to grow new tissues from isolated cells is a basic principle in tissue engineering. This strategy is emerging as a significant potential alternative or complementary solution, whereby tissue and organ failure is addressed by implanting natural, synthetic, or semi-synthetic tissue and organ mimics that are fully functional from the start or that grow into the required functionality. Numerous tissue-engineering approaches involve 3D collagen scaffolds [1] due to their major advantages of being biodegradable, biocompatible, easily available and can be processed in a variety of forms [2,3]. Therefore collagen scaffolds have been used in numerous medical applications: drug delivery, hemostatic pads, skin substitutes, soft tissue augmentation, suturing and as tissue engineering substrate [4–6].

The bone repair and regeneration are extensively studied areas in the field of tissue engineering [7]. Most of the current tissue engineering approaches to create bone substitutes focus on the development of biohybrid scaffolds composed by cells and matrices with three-dimensional architecture. Using this technology, tissue loss can be substitute by

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implantation of an engineered biohybrid structure in order to generate the required tissue, to be furter transplanted to the needed location; this construct should be functional at the time of implantation and to have the potential to integrate and form the bone tissue at a later stage. Three dimensional (3-D) bio-degradable biohybrid scaffolds are the basis of bone tissue engineering, where the specific osteoprogenitor cells can grow, multiply and differentiate into osteoblasts in a structure similar to bone tissue in the living body [8].

Osteoblast differentiation is an essential part of bone formations that compensates bone resorbtion by osteoclasts and maintain its structural integrity. Mesenchymal stem cells (MSCs) hold promise for bone healing because of their capacity to differentiate into osteoblasts and their availability from a wide variety of sources. Bone marrow represents one of the most used sources of MSCs [9,10]. It has been reported that several systemic and local hormones, growth factors and cytokines are involved in the osteoblast differentiation process both in vitro and in vivo [11]. Thus supplementation of the culture medium of MSCs with ascorbic acid, dexamethasone (dexa), beta-glycerophosphate (b-GlyP) and 1,25-dihydroxyvitamin D3 can induce the appearance of several osteoblast features [12–15].

We report here the effect of collagen—dexamethasone (Dex) and collagen −1, 25 dihydroxycholecalciferol scaffolds (D3) on the adhesion, proliferation and modulation of bone specific genes of two cell lines: an osteosarcoma cell line (MG63) and human osteoblast precursor cell line (hFOB1.19).

Materials and Methods

Collagen Scaffolds Preparation

Type I fibrillar collagen gel having a concentration of 1.2% (w/w) was extracted from calf hide by the currently used technology in INCDTP, Division ICPI-Collagen Department [16]. Glutaraldehyde was purchased from Merck, Germany. Sodium hydroxide and phosphate buffer solution (PBS, pH = 7.4) were of analytical grade. The pH of collagen gel was adjusted at 7.4 under mechanical stirring with 1 M sodium hydroxide. Dexamethasone 10^{-7} M, on one side and 1,25 dihydroxicholeocalciferol (D3 vitamin), 10^{-7} M, on the other side were embedded into collagen gel and then collagen references, collagen with Dex and collagen with D3 vitamin gels were cross-linked with 0.25% glutaraldehyde (GA) (reported to dry substance) at 4°C during 24 hours. The gels were then freeze-dried using the Christ Model Delta 2–24 KD lyophilizer, Germany, to obtain 3D collagen scaffolds (spongious forms).

In Vitro Colonization of Scaffolds

For in vitro colonization we used the human osteosarcoma cell line MG 63 and human osteoblast precursor cell line hFOB 1.19. The MG 63 cells were grown in DMEM nutrient medium containing 1‰ glucose, 10% heat inactivated fetal bovine serum, and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin, 50 U/ml neomycin) and h FBO 1.19 cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum and 0.3 mg/ml geneticine (G 418). Collagen-Dex and Collagen-D3 scaffolds were sterilized for 24 hours with 70% ethanol, and then conditioned in the same culture medium for 24 hours and inoculated with two cell lines mention above at a density of 50.000 cells/ml. The cells on scaffolds were maintained in culture at 37°C in incubators with 5% CO_2 in air

(v/v) and relative humidity over 95%. All experiments were done after 1 week of culture. Chemicals and antibiotics used for cell cultures were obtained from Sigma (Germany) and tissue culture plates from TTP (Switzerland).

Confirmation of Scaffolds Colonization

After one week in culture, the cells on collagen scaffolds were washed in PBS, fixed in 2% paraformaldehyde and then cryoprotected. Specimens were frozen in liquid nitrogen and sectioned with a Leica CM 1800 cryotome; the thickness of the sections was 4–6 mm. The cryosections were washed with PBS for 15 minutes, and stained with the nuclear dye, Hoechst 33342 (0.2 μg/ml) for 15 min, washed in distilled water, mounted in glycerol and examined with a Nikon microscope equipped with epi-fluorescence; the micrographs were captured with a Sony DSC-S75 Digital Camera.

Scanning Electron Microscopy Characterization

Osteosarcoma and osteoprecursor cells morphology on colonized scaffolds were fixed with 2.5% v/v glutaraldehyde in 0.1% w/v cacodilat buffer (pH 7.4) for 1 hour, rinsed in 0.1% v/v phosphate-buffered solution containing 0.1 M sucrose, (3 $\times$ 5 minutes) and postfixed in 1% w/v osmium tetroxide in 0.1% v/v phosphate buffer for 1 hour. Specimens were dehydrated in graded ethanol series, critical point dried, and gold-sputtered prior to observation series, critical point dried, and gold-sputtered prior to observation. The cells morphology on populated scaffolds was assessed with a Philips XL-30-ESEM TMP apparatus at an accelerating voltage of 25 kV.

Cell Viability

Cell viability was determined by MTT (Sigma Germany) assay—a colorimetric method for the determination of cell densities. The MTT is a reliable assay method for measuring cell viability in different scaffolds [17]. The assay is dependent on the cleavage of the yellow tetrazolium salt to the purple formazan crystals by metabolic active cells [18]. The cells cultured on collagen scaffolds (disks 5 mm in diameter and 2 mm thick) were incubated with 0.5 mg/mL of MTT during the last 4 h of the culture period tested; the medium was then decanted, formazan salts were dissolved with 0.1 N HCl in anhydrous isopropanol and the optical density of the formazan solution was read on a TECAN 24-well plate reader. As negative control we used collagen with gentian bleu scaffolds. The results were expressed as viability percentage.

Semiquantitative RT-PCR Analysis of Gene Expression

Total RNA was isolated from MG63 and FOB cell lines using the RNeasy Micro kit (Qiagen). The concentration and quality of RNA was determined by spectrophotometry. cDNA was synthesized from 1 μg total RNA by using oligo(dT) primers and the M-MuLV Revers Transcriptase from Fermentas. RT-PCR was performed with a kit from Qiagen (according to manufacturer's protocol) with the following primers: OC, 5′-CCTCACACTC CTCGCCCTATTG-3′ and 5′-GGTCAGCCAACTCGTCACAGTC-3′ ON, 5′-TTTGGAT GGTTTGTTGTTCTGCC-3′ and 5′-TTCCCACGGCTGTCCCAATC-3′; BSP I, 5′-TGCA GCCTTCTCAGCCAAACG-3′ and 5′-TTCCCACGGCTGTCCCAATC-3′; BSP II, 5′-AA GAGGAGGAAGAGGAAGGAAATG-3′ and 5′-GACCATCATAGCCATCGTAGCC-3′;

GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA -3′.

PCR products were resolved on 1.5% agarose gels and visualized by staining with ethidium bromide. Quantification of RT-PCR products was carried out using Scion Image software (Pharmacia Biotech, Uppsala, Sweden). The expression of OC, ON, BSP I and BSP II was normalized to a GAPDH level.

Results and Discussion

Scaffold Colonization

The potential of the scaffolds for cell colonization was tested in a conventional static cell culture system using human osteoblast-like MG 63 cells and human osteoblast precursor cell hFOB 1.19. To evaluate cellular colonization on collagen-Dex and coll-D3 scaffolds we performed Hoechst staining. We observed that all scaffolds tested allow the cells not only to attach but also to migrate inside and colonize the scaffold to form 3D structure (Figs. 1 and 2).

Cells Morphology

In order to investigate the porosity of the scaffolds and the distribution and morphology of MG63 and hFOB1.19 cells, observations by scanning electron microscopy (SEM) were performed after 7 days of culture.

All scaffolds tested present uniform porous structure with regular pores interconnected through a multitude of collagen fibers. The pores diameters are between 50 and 200 μm (Figs. 3a,b,c). After 7 days in culture the MG63 cells and hFOB 1.19 cells were completely spreaded along the available surface of the all collagen scaffolds inner pores and formed multi-layers (Figs. 3d,e,f and 4a,c) exept hFOB 1.19 cells grown on collagen—dexamethasone scaffolds (Fig. 4b).

The majority of cells showed a flat and spindle-shape and appeared in close contact one to the other forming continuous multilayer (Fig. 3a). Also, numerous cytoplasmic protrusions towards the substrate, and also between cells, were clearly visible (Figs. 3d,e,f and 4c). These findings suggest a significant increase in cell-cell interactions and a good affinity of MG63 and hFOB 1.19 cells towards this type of collagen scaffolds.

Viability of MG63 and hFOB 1.19 Cells

To explore the survival and activity of osteosarcoma cells (MG63) and human osteoblast precursor cells (hFOB1.19) grown in different collagen scaffolds for seven days, the MTT assay was used to quantify cell viability. MTT test results were given in Figs. 5 and 6. It was observed that there were no significant differences in cell viability between the scaffolds tested. However, a small decrease in viability of hFOB 1.19 cells grown on collagen-dexamethasone (Dex) was observed. This result was confirmed by SEM analysis.

Gene Expression of Bone Specific Markers in MG63 and hFOB1.19 Cells

Semiquantitative RT-PCR analysis showed that MG63 cells express high levels of osteonectine (ON), osteocalcin (OC) and bone sialoglycoprotein II (BSPII) transcripts revealed on samples grown for seven days on collagen-dexamethasone (Dex) and collagen-D3 (D3)

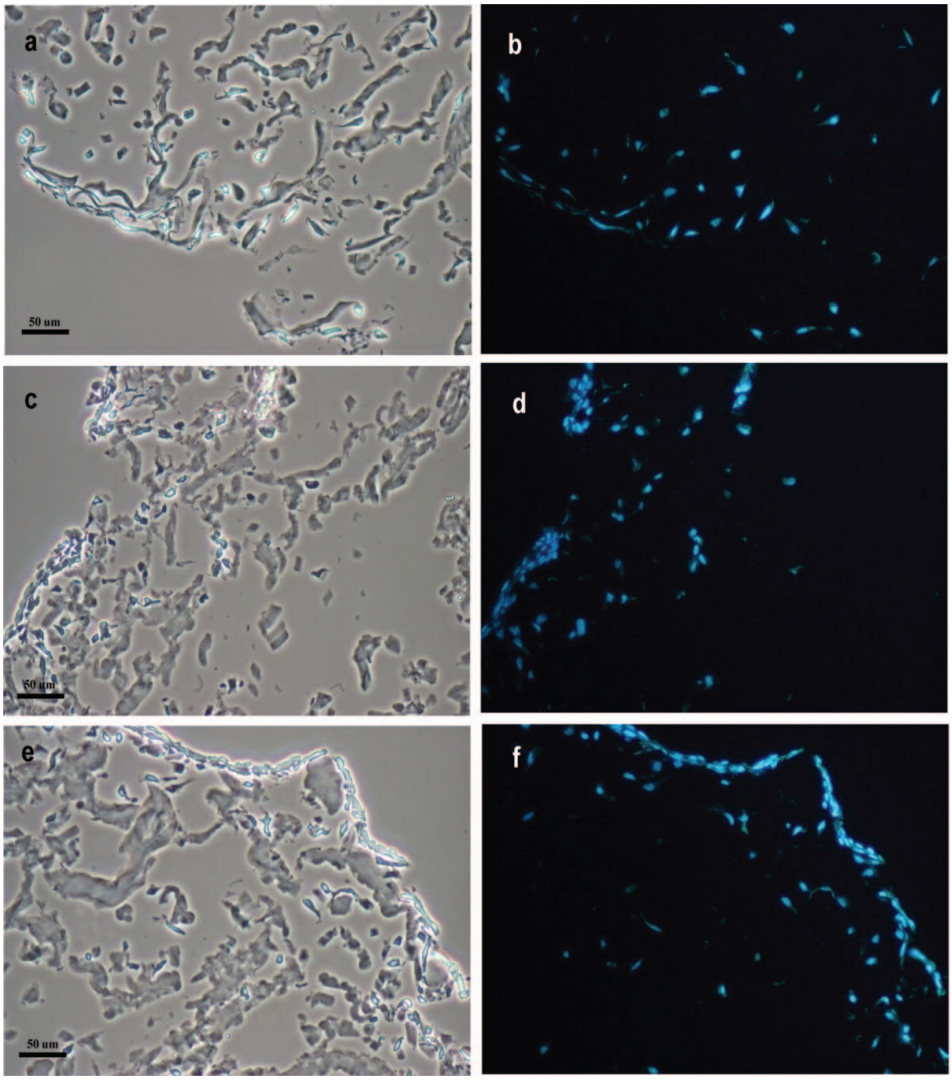

Figure 1. Micrographs of human osteoblast-like MG 63 cells on collagen scaffolds after seven days in culture: left panels—phase contrast microscopy; right panels—blue fluorescent staining with Hoechst 33342 identifying osteosarcoma cell nuclei observed by epifluorescence microscope.

scaffolds (Fig. 7b,c,d). BSP I gene expression was not observed because osteosarcoma cells do not express this marker. A representative gel with RT-PCR products obtained from RNA of cells grown on borosilicate glass (Fig. 7a—lane 1), collagen-Dex (lane 2), collagen-D3 (lane 3) and collagen control (lane 4) scaffolds for seven days was illustrated in Fig. 7a. In osteonectin gene expression no significant difference was noticed in cells grown on borosilicate glass and control collagen scaffolds compare with cells grown on collagen-Dex and collagen-D3 scaffolds (Fig. 7b). Exposure of cells to collagen-Dex and collagen-D3 scaffolds increased the gene expression of osteocalcin (Fig. 7c). Compare with cells grown on controls (borosilicate glass and collagen scaffolds) BSP II gene expression increased in cells on collagen-Dex and collagen-D3 scaffolds (Fig. 7d). All the data are normalized to

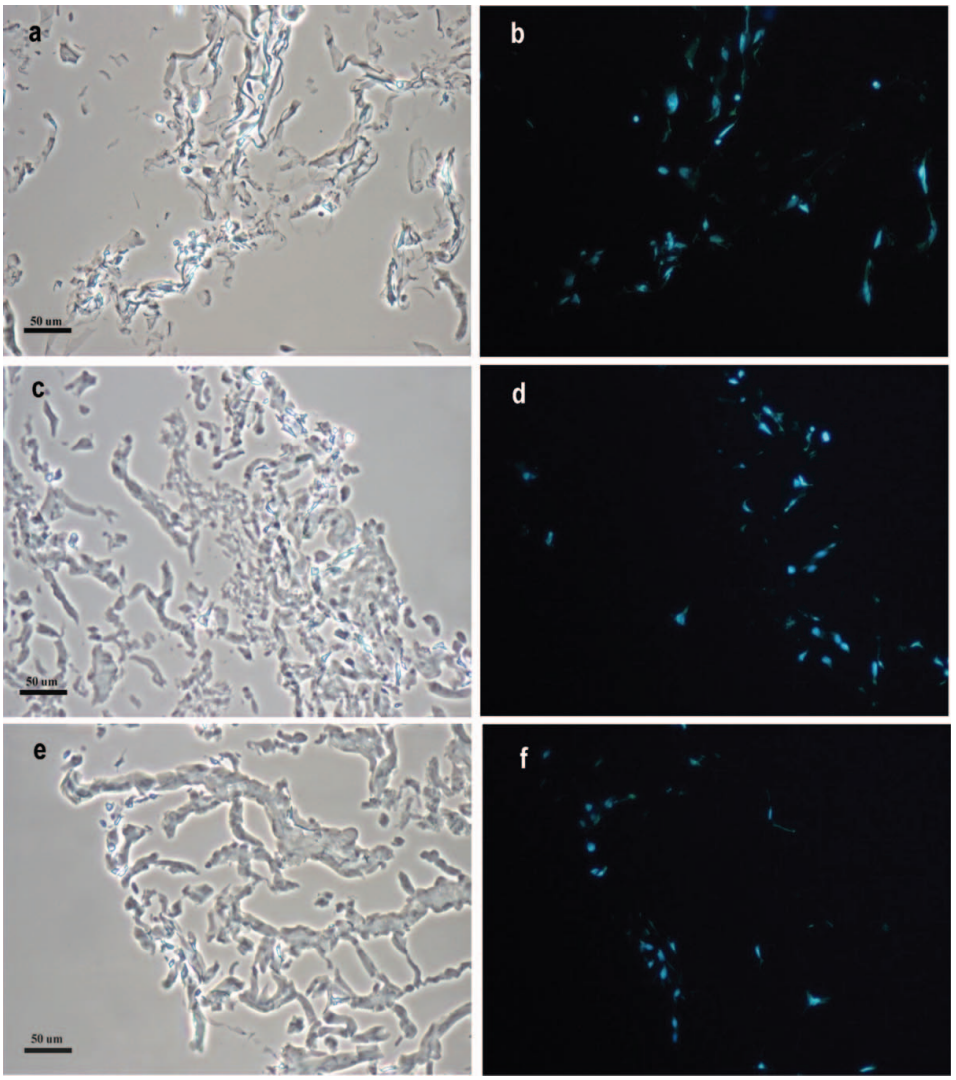

Figure 2. Micrographs of human osteoprogenitor h FOB 1.19 cells on collagen scaffolds after seven days in culture: left panels—phase contrast microscopy; right panels—blue fluorescent staining with Hoechst 33342 identifying osteoprogenitor cells nuclei observed by epifluorescence microscope.

GAPDH expression, which was considered as 1 relative unit. Data representative of two independent experiments are shown in this paper.

These results provide evidence that collagen-dexamethasone and collagen-D3 scaffolds promote osteogenic activity of MG 63 osteosarcoma cells.

The osteonectin gene expression in hFOB 1.19 cultures for seven days on collagen-Dex and collagen-D3 scaffold slightly decreased as compared with collagen control scaffolds (Fig. 8b). The gene expression of bone sialoglycoprotein I decreased in cells cultured on collagen-Dex and collagen-D3 collagen scaffolds. By contrast, the BSP II gene was weakly expressed in cells grown on borosilicate glass and collagen control scaffolds and increased in cells culture on collagen-Dex and collagen-D3 scaffolds (Fig. 8d). Osteocalcin

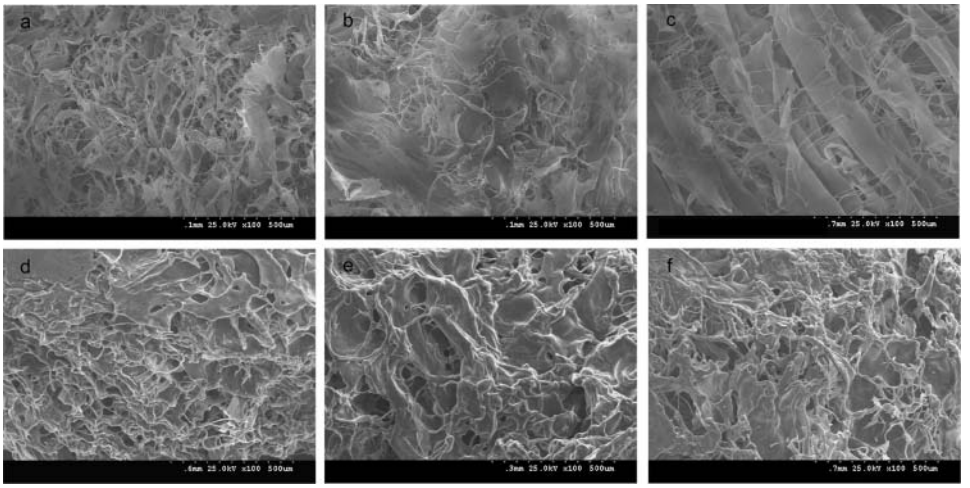

Figure 3. MG63 cells grown for seven days on collagen scaffolds: (a) collagen scaffold, (b) collagen dexamethasone scaffold, (c) collagen D_3 scaffold, (d) MG63 cells on collagen scaffold, (e) MG63 cells on collagen dexamethasone scaffold, (f) MG63 cells on collagen D_3 scaffold.

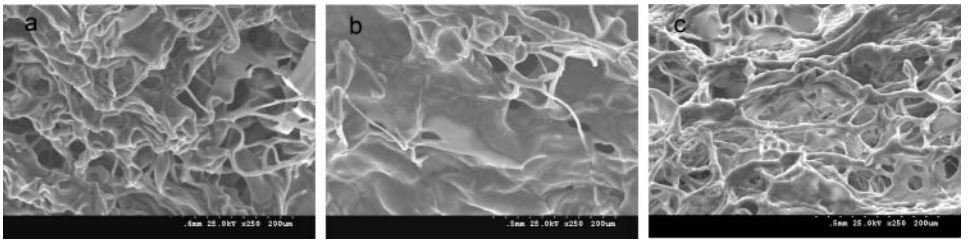

Figure 4. hFOB1.19 cells grown for seven days on collagen scaffolds: (a) hFOB1.19 cells on collagen scaffold, (b) hFOB1.19 cells on collagen dexamethasone scaffold, (c) hFOB1.19 cells on collagen D_3 scaffold.

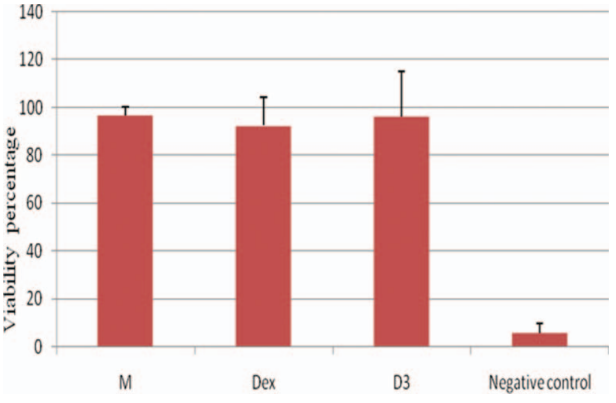

Figure 5. Oxidative metabolism of MG63 cells within collagen scaffolds: The MTT values were not significantly different when the Dex and D3 scaffolds were compared with collagen scaffold (M).

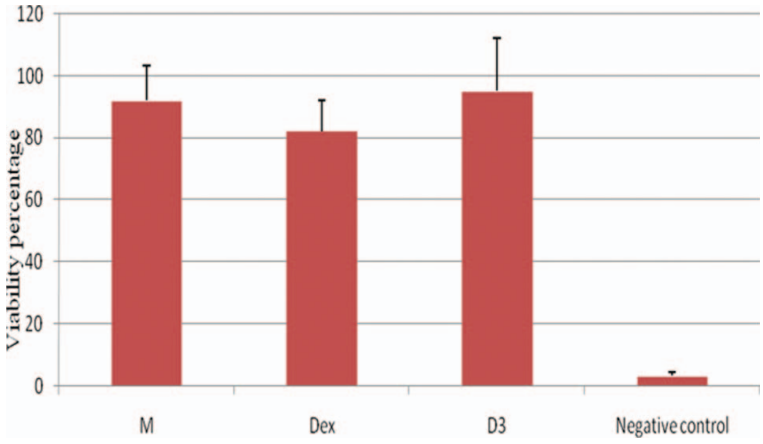

Figure 6. Oxidative metabolism of hFOB 1.19 cells within collagen scaffolds: Statistically significant differences were not found when the MTT values obtained from collagen-dexamethasone (Dex) and collagen D3 (D3) scaffolds were compared to collagen scaffolds (M). A slight decrease in viability of cells grown on collagen-dexamethasone (Dex) was observed.

gene expression was almost undetectable in cells grown on collagen scaffolds (Fig. 8a). It is known that osteonectin is expressed by preosteoblasts and osteoblasts before the expression of osteocalcin. Osteocalcin is a late and specific marker and is produced by osteoblats just before and during matrix mineralization [19]. Osteocalcin absence may be explained by the

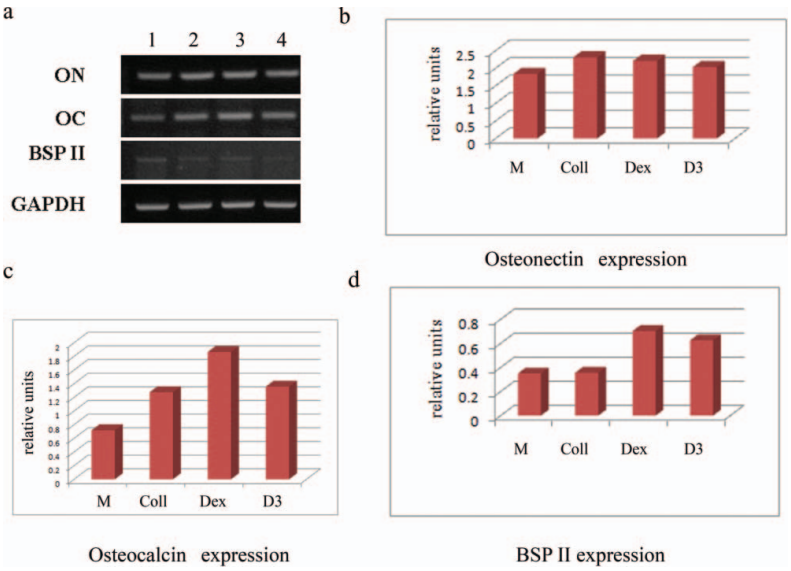

Figure 7. Analysis of osteonectin, osteocalcin and BSP II gene expression in MG63 cells grown for 7 days on different collagen matrices assessed by RT-PCR (1) borosilicate glass (M), (2) collagen scaffolds (Coll), (3) collagen-dexamethasone scaffolds (Dex), (4) collagen—D3 scaffolds (D3) (a) A representative gel shows the level of expression of osteonectin, osteocalcin, and bone sialoglycoprotein II (BSP II), Densitometric quantification of gene expression of osteonectin (b), osteocalcin (c) and BSP II gene expression (d).

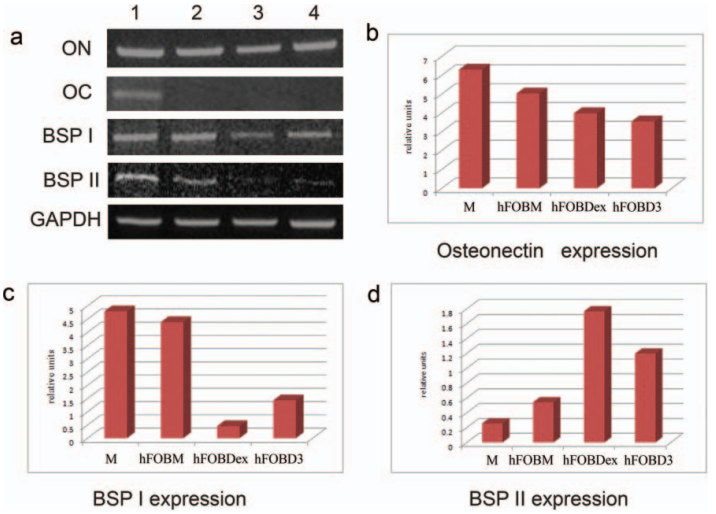

Figure 8. Analysis of osteonectin, osteocalcin, BSPI and BSP II gene expression in hFOB1.19 cells grown for 7days on different collagen matrices, assessed by RT-PCR (1) borosilicate glass (M), (2) collagen scaffolds (Coll), (3) collagen-dexamethasone scaffolds (Dex), (4) collagen-D3 scaffolds (D3); (a) A representative gel shows the level of expression of osteonectine, osteocalcin, and bone sialoglycoprotein II (BSP II). Electrophoretic RT-PCR analysis of osteonectin (b), osteocalcin (c) and BSP II gene expression (d).

fact that hFOB 1.19 cells probably required longer intervals of stimulation (culture on Dex and D3 scaffolds) to reach similar levels of expression as MG63 cells. BSP I gene expression was weak in cells grown on collagen-Dex and collagen-D3 scaffolds as compared with the expression in cells grown on controls (Fig. 8c). BSP II gene expression increased in cells grown on collagen-Dex and collagen-D3 scaffolds (Fig. 8d). GAPDH expression was used as housekeeping gene and was found to remain constant. All the data are normalized to GAPDH expression, which was considered as 1 relative unit. Data representative of two independent experiments are shown in this paper.

Conclusions

The capability of different 3D collagen scaffolds to promote the expression of some genes, significant for bone mineralization was evaluated in vitro in two human osteoblast cell lines (MG63 and hFOB1.19).

We demonstrated that human osteosarcoma MG63 cells and human osteoblasts precursor cells hFOB1.19 show a favorable and comparable viability in terms of cell growth morphology and colonization rate. Thus, collagen–Dex and collagen–D3 scaffolds promote cell adherence and development. However, the expression of bone specific markers: osteonectine, osteocalcin and bone sialoglycoprotein I and II (BSP I and II) was different in these two cell line. MG 63 cells grown for seven days on collagen–Dex and collagen–D3 scaffolds presented higher levels of osteocalcin and BSPII transcripts compared to hFOB 1.19. Moreover, the gene expression of bone sialoglycoprotein I in human osteoblasts precursor cells cultures for seven days on Dex and D3 collagen scaffolds decreased while the BSP II gene expression increased. The osteonectin gene expression in hFOB 1.19 cultured

for seven days on collagen-Dex and collagen-D3 scaffold decreased slightly as compared with collagen control scaffold.

We conclude that collagen-Dex and collagen-D3 scaffolds promote osteogenic activity of MG63 cell line while on hFOB 1.19 cells induces a decrease of gene expression of bone specific markers; it is possible that these cells require a longer period of culture on these scaffolds.

Acknowledgments

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