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Demineralized bone promotes chondrocyte or osteoblast differentiation of human marrow stromal cells cultured in collagen sponges

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Abstract

Demineralized bone implants have been used for many types of craniomaxillofacial, orthopedic, periodontal, and hand reconstruction procedures. In previous studies, we showed that demineralized bone powder (DBP) induces chondrogenesis of human dermal fibroblasts in a DBP/collagen sponge system that optimized interactions between particles of DBP and target cells in cell culture. In this study, we test the hypothesis that DBP promotes chondrogenesis or osteogenesis of human marrow stromal cells (hMSCs) in 3-D collagen sponge culture, depending upon the culture conditions. We first confirmed that hMSCs have chondrogenic potential when treated with TGF-β, either in 2-D monolayer cultures or in 3-D porous collagen sponges. Second, we found that DBP markedly enhanced chondrogenesis in hMSCs in 3-D sponges, as assessed by metachromasia and expression of chondrocyte-specific genes *AGGRECAN, COL II*, and *COL X*. Human dermal fibroblasts (hDFs) were used to define mechanisms of chondroinduction because unlike hMSCs they have no inherent chondrogenic potential. *In situ* hybridization revealed that hDFs vicinal to DBPs express chondrocyte-specific genes *AGGRECAN* or *COL II*. Macroarray analysis showed that DBP activates TGF-β/BMP signaling pathway genes in hDFs. Finally, DBP induced hMSCs to express the osteoblast phenotype when cultured with osteogenic supplements. These studies show how culture conditions can influence the differentiation pathway that human marrow stromal cells follow when stimulated by DBP. These results support the potential to engineer cartilage or bone *in vitro* by using human bone marrow stromal cells and DBP/collagen scaffolds.

Abbreviations: 3-D – three-dimensional; DBP – Demineralized Bone Powder; DMEM – Dulbecco's Modifed Eagle's Medium; FBS-HI – Fetal Bovine Serum–Heat Inactivated; hDFs – human dermal fibroblasts; hMSCs – human marrow stromal cells; IRB – Institutional Review Board.

Introduction

Autograft, allograft, and synthetic bone graft substitute materials are important treatment options in reconstructive surgery, and understanding the biologic effects of these materials is necessary for optimum implant design and use (Glowacki and Mulliken 1985; Gamradt and Lie-

berman 2003). We have used demineralized bone implants for many types of craniomaxillofacial, orthopedic, periodontal, and hand reconstruction procedures (Glowacki et al. 1981; Sonis et al. 1985; Upton and Glowacki 1992; Rosenthal et al. 1999). Animal studies showed that bone development induced by demineralized bone powder (DBP) implanted intramuscularly or subcutaneously is endochondral and similar to that seen in embryonic endochondral bone formation and adult fracture repair (Urist 1965; Reddi and Huggins 1972).

In previous studies, we showed that DBP induces chondrogenesis in a DBP/collagen sponge system that optimized interactions between particles of DBP and target cells in cell culture (Mizuno and Glowacki 1996a, b). In that system, DBP induces chondrogenesis in human dermal fibroblasts (hDFs), as demonstrated by histochemical, biochemical, and molecular markers of cartilage (Mizuno and Glowacki 1996a; Glowacki et al. 1998). When DBP/collagen sponges were implanted subcutaneously in animals, chondroinduction occurred vicinal to the particles of DBP (Mizuno and Glowacki 1996b). Human bone marrow stromal cells (hMSCs) have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, and muscle (Pittenger et al. 1999). Controlling the differentiation of hMSCs could be a useful approach to treat or prevent skeletal diseases (Nuttall and Gimble 2000).

In this study, we tested the hypothesis that DBP stimulates chondrogenesis or osteogenesis of human marrow stromal cells (hMSCs) depending upon the culture conditions. Differentiation was evaluated with tissue-specific histochemical, biochemical, and RT-PCR methods. Studies on the mechanisms of *in vitro* chondroinduction used *in situ* hybridization and macroarray analyses.

Materials and methods

Porous collagen sponges

Porous 3-D collagen sponges (8-mm diameter) were prepared from pepsin-digested bovine collagen (Mizuno and Glowacki 1996a, b). In brief, 250 μl of 0.5% collagen solution (Cellagen PC-5, ICN Biomedicals, Costa Mesa, CA) was neutralized with 1 M HEPES (pH 7.4) and 1 M $NaHCO_3$, poured into a mold, frozen, lyophilized, and irradiated with ultraviolet light. Control sponges consisted of a single layer (1.5-mm) of collagen. DBP was prepared from rat long bones (Glowacki 1996). Bilaminate collagen sponges were prepared by placing a spacer of moistened paper between two layers (0.7-mm each) of collagen. After irradiation of the sponges, DBP (3 mg) was inserted between the two layers. Sponges were transferred to seeding chambers (Mizuno and Glowacki 1996).

Cells and culture conditions

Skin samples were surgically discarded material (neonatal foreskin) obtained under IRB approved protocols (Mizuno and Glowacki 1996). Primary human dermal fibroblast (hDFs) were established by outgrowth from minced tissue (1-mm^3 pieces) and were expanded *in vitro* with DMEM (Invitrogen, Carlsbad, CA), containing 10% FBS (Invitrogen) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen), and used between 5 and 12th passages. Suspensions of hDFs were seeded onto DBP/collagen and control collagen sponges in seeding chambers. The cells (1×10^6 cells in 50 μl per sponge) were deposited directly on top of the dry sponges. The seeded sponges were placed into a humidified chamber at 37 °C with 5% CO_2 in air for 1 h. An additional 50 μl of culture medium was added to each seeded sponge and they were returned to the incubator for 2 h for hydration. The seeding chambers were transferred to 12-well tissue culture plates with 4 ml of culture medium and were tilted to ensure that no air bubbles remained beneath the sponges. The medium was changed every 3 days. The sponges were removed from the seeding chambers for RNA (n = 12 sponges in each group) after 3 days, and for *in situ* hybridization (n = 3 sponges in each group) after 7 days.

Human bone marrow stromal cells were prepared as described (Zhou et al. 2004a). In brief, femoral bone marrow was obtained as discarded material from 37-, 42-, 58- and 69-year-old women undergoing total hip replacement for osteoarthritis. Low-density mononuclear cells were isolated by density centrifugation on Ficoll/Histopaque 1077 (Sigma, St. Louis, MO). Adherent human marrow stromal cells (hMSCs)

were expanded in 2-D monolayer culture with phenol red-free MEM-α medium, 10% Fetal Bovine Serum-Heat Inactivated (FBS-HI) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen). For chondrogenesis, passage 3–6 h MSCs were seeded in 3-D collagen sponges or DBP/collagen sponges (2 million cells per sponge). After 3 days culture in MEM-α supplemented with 100 U/ml of Pen-Strep and 10% FBS-HI, the medium was changed to serum free MEM-α with 1% ITS^{+1} (Sigma; 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml BSA, 4.7 μg/ml linoleic acid), 10^{-8} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen). The sponges were harvested for histological staining and RNA at day 21. For osteogenesis, hMSCs (one million cells per sponges) were cultured in MEM-α with 10% FBS-HI and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen) plus 10^{-8} M dexamethasone, 5 mM β-glycerophosphate, and 50 μg/ml ascorbate-2-phosphate (Sigma). Sponges were harvested for biochemical alkaline phosphatase (ALP) assays and RNA after 21 days culture in osteogenic medium. For the effects of TGF-β on hMSCs, low-density i.e., undifferentiated mononuclear bone marrow cells (58-year-old woman) were cultured in 2-D monolayer cultures (10×10^6 cells per 100 mm dish) or 3-D porous collagen sponges (40×10^6 cells per sponge). Culture medium was Ham's F-12/DMEM (high glucose, 50/50 volume), 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 14 days, medium was changed to 1% FBS, 130 μM ascorbic phosphate, and 100 nM dexamethasone, with/without 10 ng/ml TGF-β1 (R & D systems, Minneapolis, MN). Sponges were harvested for histological analyses at day 14, 25, 35, and for RNA at day 35. Cells in 2-D monolayer cultures were harvested for RNA at day 35.

Histological analysis

One sponge from each group was fixed in 0.1 M cacodylate buffer containing 2% paraformaldehyde, pH 7.4, at 4 °C for 24 h. After being rinsed in 0.1 M cacodylate buffer, they were embedded in glycolmethacrylate (JB-4; Polysciences, Warrington, PA). Cross-sections (10 μm-thick) were cut in the central region of the sponges and were stained with 0.5% toluidine blue-O, pH 4.0 (Fisher Scientific, Pittsburgh, PA).

ALP biochemical assay

After 21 days culture in osteogenic medium, ALP activity in hMSCs in collagen and DBP/collagen sponges was measured colorimetrically. Sponges were homogenized with a Kontes' Pellet Pestle in lysis buffer containing 150 mM NaCl, 3 mM $NaHCO_3$, 0.1% Triton X-100, and a mixture of protease inhibitors (Roche, Mannhem, Germany). Insoluble cell materials were separated by centrifugation at 16,000$\times$ g in a microcentrifuge. Protein concentration was determined with the BCA system (Pierce, Rockford, IL). ALP enzyme activity was measured colorimetrically with a microplate reader (Model 550, Bio–Rad, Cambridge, MA). In brief, 50 μl of the supernatant was mixed with the same volume of lysis buffer containing 10 mM p-nitrophenylphosphate (Sigma) in the wells of a 96-well plate. After 30 min at 37 °C, the reaction was stopped with 3 M NaOH (Sigma) and the absorbance was measured at 405 nm. ALP activity was calculated from a standard curve with calf ALP enzyme (Promega, Madison, WI). One unit of ALP is defined by hydrolysis of 1 μmol of p-nitrophenylphosphate per minute at pH 9.8 at 37 °C. The ALP activity was shown as μmol/min/g protein after normalizing to protein concentration.

RNA isolation and RT-PCR

Total RNA was isolated from hMSCs or hDFs with Trizol reagent (Invitrogen). For RT-PCR, 2 μg of total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen), following the manufacturer's instructions. One-tenth of the cDNA was used in 50 μl PCRs (30–35 cycles of 94 °C for 1 min, 55–60 °C for 1 min, and 72 °C for 2 min) with gene-specific primers. Primers for *AGGRECAN* and *COLLAGEN TYPE II* (Glowacki et al. 1998), *COLLAGEN TYPE I, OSTEOCALCIN* and *ALP* (Lomri et al. 1999) were used for amplification.

In situ hybridization

After 7 days culture of hDFs in collagen or DBP/collagen, the sponges were harvested and fixed in RNAse-free PBS containing 4% paraformaldehyde (pH 7.5) at 4 °C overnight. After dehydration, sponges were embedded in paraffin and sections were cut at a thickness of 4 μm. Sections were deparaffinized and dehydrated before the *in situ* procedure. Digoxigenin-11-UTP-labeled single-strand ribo-probes were prepared with the DIG RNA-labeling kit (Roche, Indianapolis, IN) by *in vitro* transcription according to the manufacturer's protocol. Human *AGGRECAN* or *COL II*-specific antisense and sense probes were synthesized to cover 349 bp of cDNA for *AGGRECAN* (GenBank Accession no. XM_007701.3, bases 3665–4014), or 379 bp for *COLLAGEN TYPE II* (XM_012271.2, bases 1028–1408). Hybridization was carried out as described (Breitschopf and Suchanek 1996). Hybridization was detected with Fast Red (Roche) as substrate for ALP at 4 °C for 2–5 h. After counterstaining with Hematoxylin (Zymed, South San Francisco, CA), slides were allowed to dry and were mounted with an aqueous mounting solution (Zymed).

Gene array

GEArray™ Human TGF-β/BMP Signaling Pathway macroarrays (http://www. superarray. com) were purchased from SuperArray, Inc. (Bethesda, MD). Hybridization and analyses were performed as previously described (Zhou et al. 2004b). In brief, 3 μg of total RNA from hDFs was reverse-transcribed into cDNA with [$\alpha-^{32}$P]dCTP and reagents provided by the manufacturer. The labeled cDNAs were hybridized overnight to the macroarrays. They were washed and exposed to X-ray film for 1, 2, 5, 6, and 18 h to allow quantification of genes expressed at relatively high, low, and very low levels. Digital images were obtained by scanning autoradiographs with a transparency adapter (Epson). Data were extracted from the images with ScanAlyze software(version 2.50) (http://rana.lbl.gov/EisenSoftware. htm). The expression value for each gene on the array was calculated with GEArrayAnalyzer™ software (version 1.2) provided by the manufacturer, and was verified by visual comparison with the autoradiographs. Genes whose expression levels were measured on short (1 or 2 h) film exposures were classified as high expression; 5–6 h film exposures were classified as low expression; and 18 h film exposures were classified as very low expression. Quantitative data for gene expression levels in DBP/collagen sponges were expressed as the fold difference or percent difference relative to control (collagen sponges) after normalization with the most appropriate of the four potential controls on each array (glyceraldehye-3-phosphate dehydrogenase, GAPDH; peptidylprolyl isomerase A, PPIA; ribosomal protein L13a, RPL13A; and β-actin, ACTB). Qualitative measures of increase, decrease, or no change were used for genes expressed at levels too low for quantification.

Results

TGF-β1 stimulates chondrocyte differentiation of hMSCs in 2-D and 3-D cultures

We assessed the effects of TGF-β1 on chondrogenic differentiation of hMSCs in 2-D monolayer cultures and in 3-D collagen sponges. Low-density mononuclear bone marrow cells (58-year-old woman) were cultured in 2-D monolayer cultures (10×10^6 cells per 100 mm dish) or 3-D porous collagen sponges (40×10^6 cells per sponge) with/without 10 ng/ml TGF-β1. Molecular analysis of cells cultured for 35 days in 2-D monolayer showed that TGF-β1 induced expression of the chondrocyte-specific genes, *AGGRECAN* and *COLLAGEN TYPE II* (Figure 1). Some 3-D

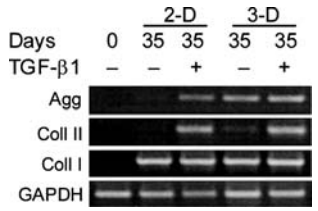

Figure 1. Effects of TGF-β on chondrocyte-specific gene expression in human marrow stromal cells (58-year-old woman). The gene expression of *AGGRECAN* and *COL II*, and *COL I* in 2-D and 3-D cultures (day 0 and day 35 after treatment) by RT-PCR. *GAPDH* was used as an internal control.

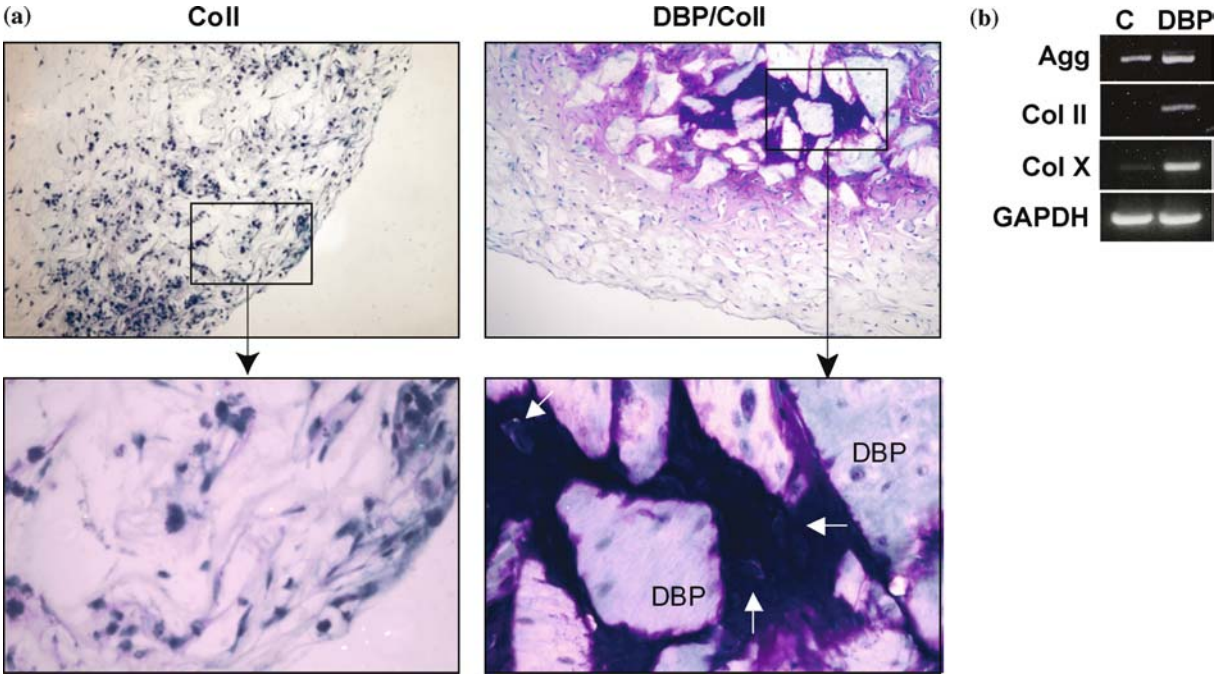

Figure 2. Effects of DBP on chondrocyte differentiation of hMSCs (42-year-old woman) in 3-D collagen sponges. a: Photomicrographs of collagen (Coll) and DBP/collagen sponge (DBP/Coll) 3 weeks after seeding with hMSCs. Upper panels show distribution of cells within the sponges. Magnification 48×. Boxed areas are shown in lower panels at higher magnification (380×). Arrows indicate chondrocyte-like cells in lacunae surrounded by metachromatic extracellular matrix (Toluidine blue stain). b: RT-PCR analysis for chondrocyte-specific gene expression of *AGG* (Aggrecan), *COL II* (Collgen type II), and *COL X* (Collagen type X) in collagen (c) and DBP/collagen (DBP) sponges. *GAPDH* was used as an internal control.

sponge cultures were harvested for histological analyses on day 14, 25, and 35. At day 14, there was an even distribution of cells throughout all sponges and little extracellular matrix as shown by staining with toluidine blue. At day 25, sponges treated with TGF-β1 showed more cellularity and accumulation of metachromatic matrix, whereas cells grown in the absence of TGF-β1 were spindle-like and those sponges showed little metachromatic matrix. At day 35, cells treated with TGF-β1 were cuboidal or round with dense accumulation of metachromatric extracellular matrix, whereas cells grown without TGF-β1 had spindle or cuboidal shape with little metachromatric matrix. Molecular analysis of hMSCs by RT-PCR showed that there was no expression of *AGGRECAN, COL I, or COL II* upon isolation of the cells (day 0). After 35 days in 3-D, TGF-β1 up-regulated the chondrocyte-specific genes *AGGRECAN* and *COLLAGEN TYPE II*, but not *COLLAGEN TYPE I* expression in hMSCs (Figure 1). Cells from 3-D cultures expressed more of these cartilage-specific markers than cells from 2-D cultures. We conclude that TGF-β1 and 3-D collagen scaffolds promote chondrocyte differentiation of hMSCs *in vitro*.

DBP promotes chondrocyte differentiation of hMSCs in 3-D collagen sponges

Effects of DBP on hMSCs in 3-D porous collagen sponges were assessed with serum-free medium. Adherent hMSCs (42-year-old woman) were seeded into DBP/collagen or control collagen sponges (2×10^6 cells per sponge). After 3 weeks, the sponges were processed for histological study or gene expression analysis. Cells that were cultured in porous collagen sponges were cuboidal in shape with little metachromatric matrix. In contrast, DBP induced hMSCs chondrocyte differentiation, as evidenced by dense accumulation of metachromatric extracellular matrix and the round shape of cells often in lacunae in proximity to DBP (Figure 2A). Furthermore, RT-PCR analysis showed that DBP up-regulated expression

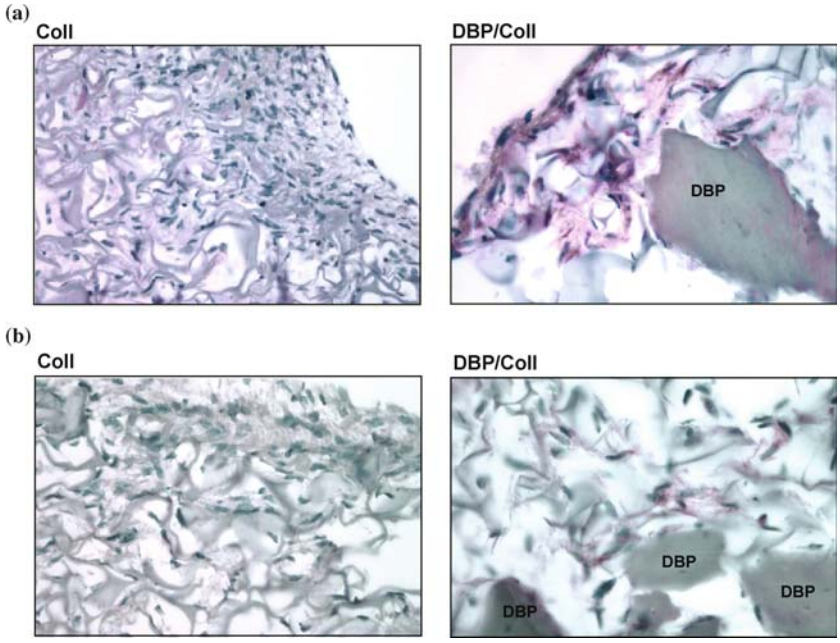

Figure 3. Photomicrographs of *in situ* hybridization for localization of a: chondrocyte-specific *AGGRECAN* or b: *COLLAGEN TYPE II* gene expression. Human dermal fibroblasts (hDFs) were cultured for 7 days in collagen (Coll) or DBP/collagen (DBP/Coll) sponges. *In situ* hybridization with DIG-labeled *AGGRECAN* or *COL II* antisense probes reveals expression of AGGRECAN and *COL II* (shown by red stain) in cells vicinal to DBP and no detectable expression in cells in collagen sponges. Hematoxylin counter-stain, magnification 370x.

of chondrocyte-specific genes, *AGGRECAN, COLLAGEN TYPE II* and *COLLAGEN TYPE X* in hMSCs (Figure 2B).

DBP induces chondrocyte-specific gene expression in human dermal fibroblasts: in situ hybridization

To localize the cells that express chondrocyte-specific genes *AGGRECAN* and *COLLAGEN TYPE II* in the presence of DBP in 3-D cultures, we used hDFs. This is because unlike hMSCs hDFs have no inherent chondrogenic potential, but are chondroinduced by DBP in collagen sponges. *In situ* hybridization was used to localize chondrocyte-specific gene expression. Human dermal fibroblasts (hDFs) were cultured for 7 days in collagen or DBP/collagen sponges. *In situ* hybridization with DIG-labeled antisense probes revealed that hDFs express *AGGRECAN* or *COLLAGEN TYPE II* in cells vicinal to DBPs in the sponges, and no detectableexpression of either gene in cells in plain collagen sponges (Figure 3). When tested with DIG-labeled *AGGRECAN* or *COL II* sense probes as negative controls, no positive signals were detected in DBP/collagen or control collagen sponges (data not shown).

DBP modulates TGF-β/BMP signaling and target genes in hDFs

We examined whether DBP regulates signaling genes in the TGF-β/BMP pathways in target cells *in vitro*. To obtain a detailed view of signaling in hDFs exposed to DBP in 3-D collagen sponges, we used a macroarray specific for TGF-β/BMP pathways (Figure 4). Control hDFs expressed some of the TGF-β superfamily receptors and intracellular signaling protein Smads, albeit at relatively low levels. The most highly expressed TGF-β/BMP signaling and target genes in control hDFs included some Smad target genes (*TGFBI/βig-h3, COL1A2, COL3A1, IGFBP3, TIMP1)* as well as signaling proteins and transcription factors (*v-JUN* and *RUNX2/CBFA1)*.

Expression profiles for hDFs were dramatically different in sponges with and without DBP. The

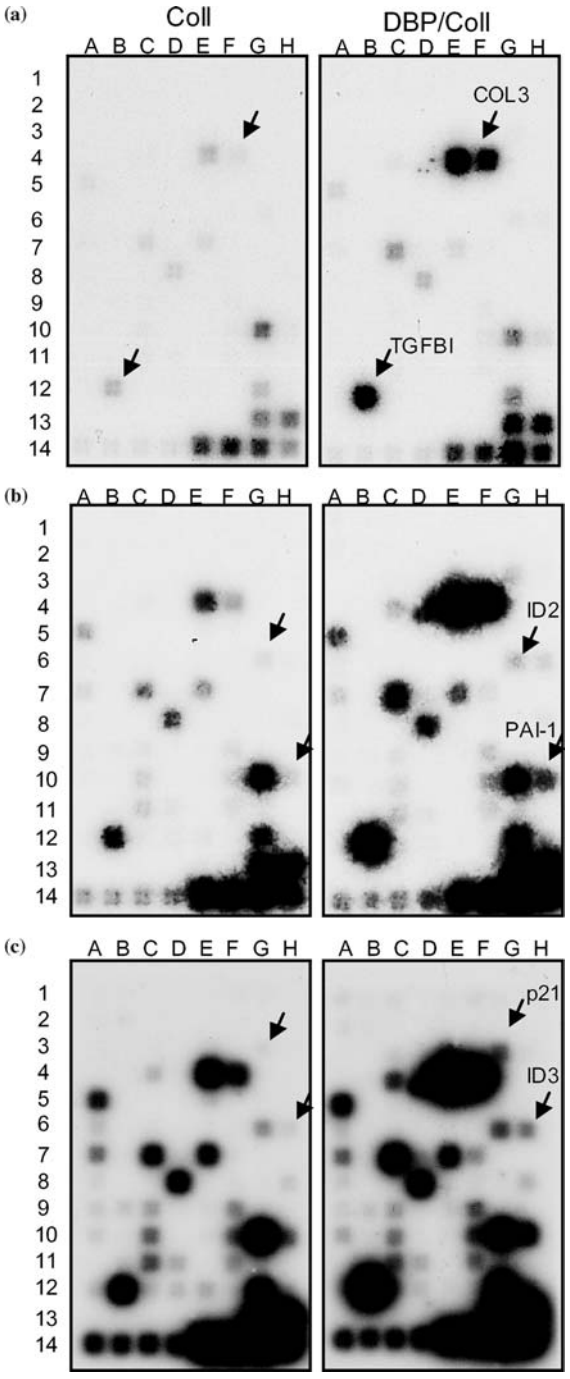

Figure 4. Effects of DBP on gene expression profiles of TGF-β/BMP signal transduction pathway in hDFs. Multiple exposures were performed a: 1–2 h; b: 5–6 h: and c: overnight and analysis were carried out with the most appropriate of the normalization controls (rows 13 and 14). Arrows indicate representative genes classified as high expression in A, low expression in B, or very low expression in C.

TGF-β/BMP signaling pathway genes that were regulated by DBP belonged to one main group, the Smad target genes, i.e. *TGFBI/βig-h3* (12.6 fold vs. collagen sponges), *COL1A2* (17.7 fold), *COL3A1* (14.0 fold), *IGFBP2* (3.8 fold), *TIMP1* (2.5 fold), *PAI-1* (4.2 fold), *p21WAF1* (2.6 fold), *ID2* (2.0 fold), *ID3* (6.4 fold), *ID4* (0.4 fold), and *STAT1* (0.5 fold). Functional classification of those target genes revealed that DBP increased expression of genes encoding several peptide differentiation factors (*TGFBI/βig-h3, IGF-BP3, Inhibin α* (1.4 fold), receptors (*Endoglin*, 1.4 fold), signaling molecules (*v -jun*, 2.0 fold) and matrix proteins (*COL1A2, COL3A1*). Increases in gene expression for other factors (*BMP1, Anti-mullerian hormone*) and receptors (*Activin receptors I, IB, II, and II-like; BMP receptor IA*) were detected but could not be precisely quantified due to very low levels in control cells (Figure 4C).

Decreases in other differentiation factor receptors (*TGFβR II and III*) were detected. It is notable that no changes in expression were found for *NODAL* (a differentiation factor) or *BMP receptors IB, or II*. Several TGF-β/BMP signaling genes (*RUNX1/AML1, RUNX2/CBFA1* (0.9 fold); *SMAD 2, 3, 4, 5, and 9*) were also not changed by DBP. Those results show that hDFs express receptors for TGF-β and for BMP, and that DBP activates TGF-β/BMP signal pathway in hDFs.

DBP induces osteogenic differentiation of hMSCs in 3-D collagen sponges

Effects of DBP on osteogenic differentiation of hMSCs were assessed in 3-D porous collagen sponges with osteogenic medium. hMSCs obtained from 37-, 42- and 69-year-old women were precultured in either DBP/collagen or collagen sponges (1×10^6 cells/sponge) for 3 days with MEM-α with 10% FBS-HI. Thereafter, they were cultured in MEM-α with 10% FBS-HI with osteogenic supplements (10^{-8} M dexamethasone, 50 μg/ml ascorbic acid phosphate, 5 mM β-glycerophosphate). After 3 weeks, the sponges were harvested for ALP biochemical assays or for gene expression analysis. DBP stimulated ALP activity in hMSCs from 37- and 69-year-old women (Table 1). In independent experiments with cells from a 37-year-old woman (Expt 1 and 2), DBP significantly increased ALP activity by similar magnitudes (1.35

Table 1. Effects of DBP on osteoblast differentiation of hMSCs in 3-D collagen sponges (3 weeks in osteogenic condition).

Donor	Expt	Sponge No. (n)[b]	ALP activity (μmol/min/g protein ± SD)		DBP/Col[c]	P[d]
			COL	DBP		
37F	1	$n = 3$	37.5 ± 2.4	50.8 ± 2.4	1.35	< 0.01
	2	$n = 3$	22.2 ± 0.1	34.5 ± 3.2	1.55	< 0.01
42F	1	$n = 1$	105.0	98.3	0.94	N/A
	2	$n = 1$	209.5	127.1	0.61	N/A
	3	$n = 3$	104.5 ± 12.6	42.0 ± 1.7	0.40	< 0.01
69F	1	$n = 1$	125.3	237.1	1.89	N/A
	2	$n = 1$	31.4	160.4	5.11	N/A

Note: Experiments done on different days in different assays for ALP.
[b] Sponge number for each group in each experiment.
[c] DBP/Col: DBP relative to collagen sponge control.
[d] Unpaired *t*-test, DBP vs. Collagen sponges in experiments with replicate sponges.
N/A: not applicable.

fold, $p < 0.01$ and 1. 55 fold, $p < 0.01$, respectively). In two small experiments with cells from a 69-year-old woman, the amount of ALP activity was greater in those cells that had been cultured with DBP. In independent experiments with cells from a 42-year-old woman, DBP appeared to decrease ALP activity. The apparent inhibition by DBP is unexplained, but may be due to a greater effect of DBP on proliferation in these cells (as indicated by total protein content per sponge, data not shown) or due to unknown systemic differences in donors.

The effects of DBP on osteocyte gene expression in hMSCs (37-year-old woman) were analyzed with RT-PCR (Figure 5). DBP upregulated expression of osteoblast marker genes *ALP* and *OSTEOCALCIN*, and had no effect on *COLLAGEN TYPE I* gene expression in hMSCs.

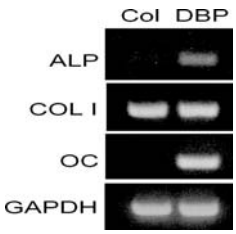

Figure 5. RT-PCR analysis of osteoblast gene expression in hMSCs (37-year-old woman) in 3-D collagen sponges (21 days). Treatment with DBP upregulated alkaline phosphatase (ALP) and OSTEOCALCIN (*OC*), but not *COL I* (Collagen type I) expression in hMSCs obtained from. *GAPDH* severed as an internal control.

Discussion

Demineralized bone implants are being used successfully in many types of craniomaxillofacial, orthopedic, periodontal, and hand reconstruction procedures (Glowacki et al. 1981; Glowacki and Mulliken 1985; Sonis et al. 1985; Upton and Glowacki 1992; Rosenthal et al. 1999). Animal studies showed that the sequential cellular changes in response to implants of demineralized bone materials include chemotaxis and attachment of progenitor cells to the matrix; proliferation and differentiation of progenitor cells into chondrocytes; sequential chondrogenesis, cartilage mineralization, vascularization, and resorption of the induced cartilage; and ultimately osteogenesis and marrow formation (Muthukumaran and Reddi 1985).

Identification of the cellular and molecular changes regulated by DBP in postnatal target cells *in vitro* should provide information about the regulation of osteo/chondrogenesis induced by DBP *in vivo*. In previous studies, we showed that DBP induces chondrogenesis of human dermal fibroblasts in a collagen/DBP sponge system that optimized interactions between particles of DBP and target cells in cell culture (Mizuno and Glowacki 1996a, b). In that system, DBP induces

chondrogenesis in hDFs, as demonstrated by histochemical, biochemical, and molecular markers of cartilage (Mizuno and Glowacki 1996a; Glowacki et al. 1998). It was unknown whether DBP induces hMSCs as well as hDFs in these 3-D DBP/Collagen sponges. Our results show that DBP induced hMSCs to differentiate to chondrocytes in serum-free conditions as demonstrated by the dense accumulation of metachromatric extracellular matrix and chondrocyte-like cells around DBP. Furthermore, DBP up-regulated chondrocyte genes *AGGRECAN, COLLAGEN TYPE II* and *COLLAGEN TYPE X* in hMSCs, as shown by RT-PCR. hDFs were used to localize cells in the sponges that became chondrocytes when cultured with DBP. *In situ* hybridization revealed that expression of *AGGRECAN* or *COLLAGEN TYPE II* in cells that were vicinal to particles of DBP, and no detectable *AGGRECAN* or *COLLAGEN TYPE II* expression in cells of control collagen sponges. These findings emphasize the importance of geometry in inductive implants (Mizuno and Glowacki 1996b)

There are many growth factors in DBP or matrix, e.g. BMPs, TGF-βs, IGFs, FGFs, PDGFs, BDGF etc. (Solheim 1998; Centrella and Canalis 1985; Kale and Di Cesare 1995). TGF-β is produced and secreted by bone cells and is stored in bone matrix (Centrella et al. 1994). Numerous experimental findings suggest that TGF-β is of primary importance in the regulation of bone turnover (Mohan and Baylink 1991; Canalis et al. 1988). TGF-β plays a significant role in promoting chondrogenesis *in vitro* and *in vivo* (Grimaud et al. 2002) and can provide competence for early stages of chondroblastic and osteoblastic differentiation, although it inhibits late-stage osteoblast differentiation *in vitro* (Roelen and Dijke 2003). Some authors posit that TGF-βs and BMPs are major osteo/chondroinduction factors in bone (Centrella et al. 1994; Wang et al. 1988). Thus it is likely that osteo/chondroinduction of DBP involves TGF-β/BMP signaling pathway genes.

We undertook an analysis of changes in gene expression after hDFs were cultured with DBP for 3 days. In a previous study, discovery-driven methods of gene expression analysis revealed several functional classes of genes (cytoskeletal and matrix elements, growth factors, and signal transduction) that are altered prior to the expression of the chondrocyte phenotype (Yates et al. 2001). A targeted macroarray was used to assess whether DBP activates TGF-β/BMP signaling pathway in target cells in our 3-D collagen scaffolds. This array can be used to screen for differences in expression of many members of the TGF-β superfamily and key proteins involved in the TGF-β signal transduction pathway, e.g. TGF-β superfamily receptors, their intracellular signaling proteins Smads, and Smad target genes. This analysis showed that DBP activated genes in both TGF-β and BMP pathways in hDFs. Although the TGF-β/Smad signaling pathways were regulated by DBP, our results show that TGF-β1 alone did not mimic the effects of DBP on human marrow stromal cells. TGF-β did stimulate chondrocyte differentiation and chondrocyte-specific gene expression of hMSCs as expected, but did not affect osteoblast gene ALP expression of hMSCs (Zhou et al. 2004a). In another study, we reported that rhBMP-2 alone did not affect all the same genes as did DBP in hDFs in 3-D collagen sponges (Zhou et al. 2004b). Thus the mechanism of induction by DBP is very complex and is not mimicked by TGF-β or by rhBMP-2. Understanding the interactions of the various growth and differentiation factors in bone matrix will be important to determine the mechanisms of DBP's action and its effects on chondroinduction or osteoinduction.

Human MSCs have been used in research for tissue engineering of bone on scaffolds. Kasten et al. used hMSCs obtained from adult donors (18–50-year-old) to compare three resorbable biomaterials for cell penetration into the matrix, cell proliferation, and osteogenic differentiation (Kasten et al. 2003). They reported that demineralized bone matrix seemed to be more favorable than calcium-deficient hydroxyapatite and β-tricalcium phosphate to support later stages of osteoblast differentiation *in vitro*. Mauney et al. found that mechanical stimulation promotes osteogenic differentiation of commercially obtained hMSCs on partially demineralized bone scaffolds *in vitro* (Mauney et al. 2004). With our porous collagen sponge system, we used hMSCs to assess whether DBP stimulates osteoblast as well as chondrocyte differentiation. Culture conditions were varied to be permissive for either cell type (Figure 6). Our results showed that DBP increased ALP activity in hMSCs obtained from 37- and 69-year-old women. Although the ALP activity in control collagen sponges and the magnitude of

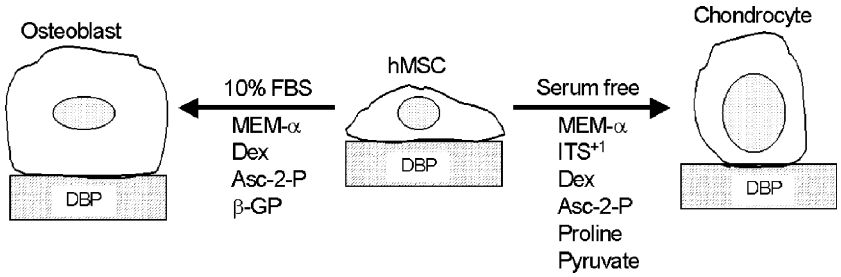

Figure 6. Effect of culture conditions on the differentiation pathways that human marrow stromal cells follow when stimulated by DBP. Chondrogenic culture conditions are MEM-α serum-free with 1% ITS^{+1}, 10^{-8} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Osteogenic culture conditions are MEM-α with 10% FBS-HI and antibiotics plus 10^{-8} M dexamethasone, 5 mM β-glycerophosphate, 50 μg/ml ascorbate-2-phosphate, 100 U/ml penicillin, and 100 μg/ml streptomycin.

DBP's effects were different in replicate experiments, there was consistency in the results using cells from each subject. The apparent inhibition by DBP in one subject (42-year-old woman) is unexplained, but may be due to a greater effect of DBP on proliferation in these cells or due to unknown systemic differences in donors. We described the age-dependent decline in osteoblast potential in hMSCs from men (Mueller and Glowacki 2001). Others noted striking differences in osteoblast differentiation in monolayer cultures of human bone marrow stromal cells treated withrhBMP-2 (Diefenderfer et al. 2003a, b). In the first of a series of comparative studies, it was shown that 18 of 19 samples of hMSCs did not increase ALP expression in response to rhBMP-2, in marked contrast to rat or mouse stromal cells (Diefenderfer et al. 2003a). There was a suggestion of an age effect because, when pretreated with dexamethasone, samples from the younger subjects showed stimulation of ALP by BMP-2 and those from older subjects did not (Osyczka et al. 2004). Our small study suggests that hMSCs from different donors may also respond differently to DBP *in vitro* in 3-D cultures under osteogenic culture conditions. Human marrow stromal cells from the oldest donor had the greatest response to DBP. More information is needed on the effects of age and other systemic factors on osteogenic differentiation of hMSCs and their response to DBP in order to evaluate the potential of using DBP for *in vitro* tissue engineering.

In summary, we first confirmed that hMSCs have chondrogenic potential when treated with TGF-β in 2-D monolayer cultures. The 3-D porous collagen sponges also supported TGF-β's stimulation of chondrocyte differentiation. Second, we found that DBP stimulated chondrogenesis in hMSCs in 3-D sponges, as assessed by metachromasia and expression of chondrocyte-specific genes *AGGRECAN, COL II, and COL X*. Human dermal fibroblasts were used to define mechanisms of chondroinduction because, unlike hMSCs, they have no inherent chondrogenic potential. *In situ* hybridization revealed that hDFs vicinal to DBPs express chondrocyte-specific genes. Macroarray analysis showed that DBP activates TGF-β/BMP signal pathway in hDFs prior to their expression of chondrocyte markers. Finally, DBP induced hMSCs to express the osteoblast phenotype with osteogenic supplements. These studies show how culture conditions can influence the differentiation pathway that human marrow stromal cells follow when stimulated by DBP. These results support the potential to engineer cartilage or bone *in vitro* by using hMSCs and DBP/collagen sponges.

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