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The Role of Retinoic Acid Receptor Inhibitor LE135 on the Osteochondral Differentiation of Human Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

The present study aimed to investigate the role of a retinoic acid receptor-β (RARβ) inhibitor LE135 on TGF-β induced chondrogenesis of human bone marrow mesenchymal stem cells (hMSCs). Pellet culture with exogenous transforming growth factor-β (TGF-β), and a mechanically loaded scaffold system were used to provide two culture models. All samples were cultured for 8 days and changes in early gene expression were determined. Glycosaminoglycan and mRNA expression data showed that LE135 itself did not induce any chondrogenic response in either pellet culture or scaffold culture of hMSCs. LE135 actually inhibited the chondrogenic response caused by exogenous TGF-β, or endogenous TGF-β induced by mechanical load, while the expression of genes normally associated with osteogenesis was not affected. This suggests that the inhibitor LE135 affects the osteochondral differentiation pathway at a different stage, inhibiting chondrogenic gene expression while having no effect on genes normally associated with the osteogenic phenotype. Alternatively, it might be that different cells were proceeding down different lineages. Some cells were undergoing chondrogenesis and this was affected by LE135, while other cells underwent osteogenic differentiation and were not affected by LE135. J. Cell. Biochem. 112: 963–970, 2011.

KEY WORDS: DIFFERENTIATION; OSTEOGENESIS; CHONDROGENESIS; RETINOIC ACID; SIGNALING

Articular cartilage is a highly specialized connective tissue that serves as a natural weight bearing material, absorbing and transmitting loads across diathroidal joints [Buckwalter et al., 2005]. However, it has a very poor ability of self-repair after damage. A number of investigations have been performed using tissue engineering due to the unsatisfactory results of the current clinical procedures. Mesenchymal stem cells derived from bone marrow have demonstrated the multipotential to differentiate into several cell lineages, including chondrocytes [Pittenger et al., 1999]. The advantage of using MSCs is that the harvest procedure is relatively easy and less destructive compared with articular cartilage harvest procedures. The drawback with the use of MSCs for articular cartilage repair is their propensity to undergo terminal differentiation into hypertrophic chondrocytes, typically expressing type X collagen and other osteogenic markers such as alkaline phosphatase [Johnstone et al., 1998].

Factors known to induce chondrogenesis of MSCs include growth factors (i.e., transforming growth factor-β [Johnstone et al., 1998], growth and differentiation factor 5 [Feng et al., 2008]) and mechanical load [Schumann et al., 2006; Li et al., 2010a; Li et al., 2010b]. However, with these growth factors and mechanical load, type X collagen is still expressed [Johnstone et al., 1998; Li et al., 2009]. Recently, Kafienah et al. demonstrated that inhibition of retinoic acid receptor-β (RARβ) with LE135 can lead to chondrogenic induction of human bone marrow derived MSCs [Kafienah et al., 2007]. This raises the potential for small molecules to modulate stem cell differentiation, which is more cost-effective and more stable compared with biological factors. Moreover, Kafienah's work reported that LE135 induced chondrogenesis without upregulation of collagen type X. The limitation of their study is that the gene expression data were from 3 days or 6 days of monolayer culture. In 3D culture, the same group demonstrated that after 21 days of culture safranin O staining could be detected in LE135 treated constructs; however, the staining was weaker than that seen with TGFβ induction [Kafienah et al., 2007]. The ability of LE135 for inducing chondrogenesis without leading to hypertrophy in 3D culture system of human bone marrow derived MSCs remains unclear.

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The pellet culture system established by Johnstone et al. is the standard for chondrogenesis of MSCs [Johnstone et al., 1998] and for this reason was included in the study to allow for comparison to previously published work. It allows cell–cell interaction similar to those that occur in precartilage condensation during embryonic development [Johnstone et al., 1998]. We have previously shown that a fibrin/polyurethane scaffold composite culture system has comparable outcome for chondrogenesis of hMSCs compared with pellet culture [Li et al., 2009]. Additionally, in the context of tissue engineering, the scaffold system supplies a structure for neo-tissue accumulation and a mechanical stiffness capable of withstanding mechanical stimuli. We, therefore, included this model system as it is possible to induce chondrogenesis, in the absence of exogenous TGF-β, by mechanical stimulation alone. These two culture systems both induce chondrogenesis through the TGF-β pathway, although there are slight differences in the final phenotype observed as can be seen in differing proteoglycan-4 gene expression [Li et al., 2010a]. The present study aimed to determine the ability of LE135 to induce chondrogenesis of human bone marrow MSCs, both in the pellet culture system and a scaffold culture system. LE135 treated samples were compared with TGF-β treated samples (standard chondrogenesis model). In the pellet system, 1 ng/ml of TGF-β1 was added to the culture medium. In the scaffold system, mechanical load was applied to the cell-scaffold constructs to induce endogenous TGF-β1 and TGF-β3 synthesis which is similar to adding 1 ng/ml exogenous TGF-β1 [Li et al., 2010a].

In this study we aimed to investigate the effect of LE135 on the early gene expression of humans MSCs stimulated towards chondrogenesis using two different mechanisms. Specifically, we were interested on the effect of LE135 on TGFβ induced chondrogenesis. The results from this study suggest that inhibition of RARβ using LE135 interferes with early fate decisions within these two culture systems. The expressions of SOX9, Col II, and Agg were all inhibited at early time points, whereas genes normally associated with an osteogenic phenotype were largely unaffected.

MATERIALS AND METHODS

ISOLATION AND EXPANSION OF HMSCS

Bone marrow was obtained with ethical approval from the local Swiss authorities and the written consent of the patients undergoing total hip replacement. MSCs from 1 male patient (67 years old) and 3 female patients (38, 78, and 81 years old) were used in the present study. The bone marrow was diluted 1:5 with Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NE) containing 5% fetal calf serum (FCS). The mixture was layered on a Ficoll cushion and centrifuged at 800 g for 20 min. Mononuclear cells were collected from the liquid interface, and washed with DMEM. The isolated cells were plated in polystyrene cell culture flasks and supplemented with α-modified essential medium (αMEM), 10% FCS, and 5 ng/ml recombinant human basic fibroblast growth factor, then cultured at 37°C, 5% CO_2, 95% humidity. The medium was changed after 3–5 days, and twice a week afterwards. P3 cells were used in three pellet culture experiments and four scaffold culture experiments. Cells obtained were tested for multi-potentiality by differentiation into adipocytes, chondrocytes, and osteoblasts (data not shown).

PELLET CULTURE OF HMSCS

P3 hMSCs were trypsinized, and 2×10^5 cell aliquots (250 μl cell suspension) were dispensed into the wells of an V-bottom 96-well 300-μl polypropylene microplate (Faust Laborbedarf AG, Schaffhausen, Switzerland). Basal culture medium consisted of DMEM, ITS + 1 (10 μg/ml insulin from bovine pancreas, 5.5 μg/ml human transferrin (substantially iron-free), 5 ng/ml sodium selenite, 0.5 mg/ml bovine serum albumin and 4.7 μg/ml linoleic acid; Sigma, St Louis, MO), 100 units/ml Penicillin (Gibco, Grand Island, NE), 100 μg/ml Streptomycin (Gibco, Grand Island, NE), 1% non-essential amino acid (Gibco, Grand Island, NE), 50 μg/ml ascorbate 2 phosphate (Sigma, St Louis, MO), and 10^{-7} M dexamethasone (Sigma, St Louis, MO). The pellets were assigned to four groups fed with different medium: group Control—pellet with basal medium; group TGFB—same medium as group Control plus 1 ng/ml recombinant human TGF-β1 (Fitzgerald, Concord, MA); group LE—same medium as group Control plus 1 μM LE135 (Tocris Bioscience, Bristol, UK); group LE + TGFB—same medium as group Control plus 1 ng/ml recombinant human TGF-β1 and 1 μM LE135. In groups lacking LE135 the equivalent amount of DMSO carrier was added. Each pellet was cultured in 0.25 ml medium, under condition of 37°C, 5% CO_2, 95% humidity. The culture medium was changed every second day and pellets were harvested on day 8. Conditioned medium was collected for biochemical analysis.

BIODEGRADABLE POLYURETHANE SCAFFOLD

Cylindrical (8 mm diameter × 4 mm height) porous scaffolds (average pore size of 90–300 μm) of biodegradable polyurethane were prepared as described elsewhere [Gorna and Gogolewski, 2006]. Briefly, the scaffolds were prepared by a salt leaching-phase inverse technique consisting of the mixing in equal weight of a porogen (sodium phosphate heptahydrate dibasic salt, particles size range from 90 to 300 μm) with a solution containing a mixture of solvents and the polyurethane synthesized from hexamethylene diisocyanate, poly(epsilon-caprolactone) diol and 1,4:3,6-dianhydro-D-sorbitol in a one step solution polycondensation reaction. The salt was leached out by extensive washing in water, and the sponge obtained washed in ethanol and dried under vacuum before cutting into 4 mm height by 8 mm diameter cylinders.

FIBRIN–POLYURETHANE COMPOSITE CULTURE OF HMSCS

A fibrin–polyurethane hybrid system was used for hMSC scaffold culture. P3 hMSCs were trypsinized, suspended in fibrinogen solution and mixed with thrombin solution immediately prior to seeding into the polyurethane scaffold at a cell density of 5×10^6 per scaffold (approximately 25,000 cells/mm^3). The fibrin components were provided by Baxter Biosurgery (Vienna, Austria). The final concentrations of the fibrin gel were 17 mg/ml fibrinogen, and 0.5 U/ml thrombin [Lee et al., 2005a]. After 1 day pre-culture in 6-well plates (5 ml medium per scaffold), cell-scaffold constructs were exposed to mechanical loading in plastic holders for 7 days as described below (3 ml medium per scaffold, medium changed everyday). The pre-culture medium consisted of DMEM, ITS,

100 units/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acid, 50 μg/ml ascorbate 2 phosphate, and 5 μM ε-amino-caproic acid (Sigma, St Luois, MO, to inhibit fibrin degradation [Kupcsik et al., 2009]). During 7 days of load, samples were assigned to two groups fed with different medium: group LE−, scaffold with pre-culture medium plus 10^{-7} M dexamethasone; group LE+, scaffold with pre-culture medium plus 10^{-7} M dexamethasone and 1 μM LE135. In groups lacking LE135 the equivalent amount of DMSO carrier was added.

MECHANICAL LOADING

Mechanical conditioning of cell-scaffold constructs was performed using our previously described bioreactor system [Wimmer et al., 2004]. Briefly, a ceramic hip ball (32 mm in diameter) was pressed onto the cell-seeded scaffold. Interface shear motion was generated by oscillation of the ball about an axis perpendicular to the scaffold axis. Superimposed compressive strain was applied along the cylindrical axis of the scaffold.

For each group fed with the same medium, samples were assigned in triplicates to one of two groups. The group of unloaded constructs served as controls. The loaded group was exposed to ball oscillation of ±25° at 1 Hz. Simultaneously, dynamic compression was applied at 1 Hz with 10% sinusoidal strain, superimposed on a 10% static offset strain, resulting in an actual strain amplitude of 10–20%. Mechanical load was performed for 1 h a day over 7 consecutive days. After 1 day in pre-culture and 7 days of load, scaffolds were vertically cut into two halves, one half for biochemical analysis, the other half for gene expression analysis.

BIOCHEMICAL ANALYSIS

Samples were digested with 0.5 mg/ml proteinase-K (2.5 U/mg, chromozyme assay, Roche, Mannheim, Germany) at 56°C overnight. DNA content was measured spectrofluorometrically using Hoechst 33258 dye [Labarca and Paigen, 1980]. The amount of GAG in the scaffolds and medium was determined by the dimethylmethylene blue dye method [Farndale et al., 1986].

GENE EXPRESSION ANALYSIS

Total RNA was extracted from pellets or homogenized scaffolds with TRI Reagent (Molecular Research Center, Cincinnati, OH). Total RNA was also isolated from cell aliquots directly after isolation from monolayer to assess their basal gene expression levels before 3D culture. Reverse transcription was performed with TaqMan reverse transcription reagents, using random hexamer primers and 1 μg of total RNA. Table I shows the designed sequence of human primers and TaqMan probes for collagens type I (COL1), type II (COL2), type X (COL10), aggrecan (AGG), osterix (Sp7), TGFB1, and TGFB3. Primers and probe for amplification of 18S ribosomal RNA (18S, Part Number 4310893E), SRY-related HMG-box gene 9 (Sox9, Hs00165814_m1), parathyroid hormone-related peptide (PTHrP, Hs00174969_m1) and AP1/Jun (Jun, Hs99999141_s1) were from Applied Biosystems (Foster City, CA). Relative quantification of target mRNA was performed according to the comparative C_T method with 18S ribosomal RNA as the endogenous control [Lee et al., 2005b].

TGF-β1 AND TGF-β3 ELISA

During 7 days of mechanical load, conditioned medium of loaded and control scaffold samples were pooled for each construct and analyzed for TGF-β1 and TGF-β3 concentrations by ELISA (DY240 and DY243, R&D Systems, Inc., Minneapolis, MN) according to manufacturers' instruction.

STATISTICAL ANALYSIS

SPSS 16.0 statistical software was used for statistical analysis (significance at $P < 0.05$). Gene expression values were normalized to the average values of cells before 3D culture (day 0). One-sample Kolmogorov–Smirnov test was used to define whether the data are normally distributed (normal distribution at $P > 0.1$). For data which

TABLE I. Oligonucleotide Primers and Probes (Human) Used for Real Time PCR

Gene	Primer	Sequence
COL1	Primer fw (5'-3')	CCCTGGAAAGAATGGAGATGAT
	Primer rev (5'-3')	ACTGAAACCTCTGTGTCCCTTCA
	Probe (5'FAM/3'TAMRA)	CGGGCAATCCTCGAGCACCCT
COL2A1	Primer fw (5'-3')	GGCAATAGCAGGTTCACGTACA
	Primer rev (5'-3')	GATAACAGTCTTGCCCCACTTACC
	Probe (5'FAM/3'TAMRA)	CCTGAAGGATGGCTGCACGAAACATAC
COL10A1	Primer fw (5'-3')	ACGCTGAACGATACCAAATG
	Primer rev (5'-3')	TGCTATACCTTTACTCTTTATGGTGTA
	Probe (5'FAM/3'TAMRA)	ACTACCCAACACCAAGACACAGTTCTTCATTCC
Aggrecan	Primer fw (5'-3')	AGTCCTCAAGCCTCCTGTACTCA
	Primer rev (5'-3')	CGGGAAGTGGCGGTAACA
	Probe (5'FAM/3'TAMRA)	CCGGAATGGAAACGTGAATCAGAATCAACT
Sp7	Primer fw (5'-3')	CCTGCTTGAGGAGGAAGTTCA
	Primer rev (5'-3')	GGCTAGAGCCACCAAATTTGC
	Probe (5'FAM/3'TAMRA)	TCCCCTGGCCATGCTGACGG
TGFB1	Primer fw (5'-3')	GACTACTACGCCAAGGAGGTCA
	Primer rev (5'-3')	GAGCTCTGATGTGTTGAAGAACATATA
	Probe (5'FAM/3'TAMRA)	TGGTGGAAACCCACAACGAAATCTATGACA
TGFB3	Primer fw (5'-3')	AATCCATAAATTCGACATGATCC
	Primer rev (5'-3')	TTGGAGGTAATTCCTTTAGGG
	Probe (5'FAM/3'TAMRA)	ACAGCCAGTTCGTTGTGCTCCG
RARβ	Primer fw (5'-3')	GGT ACC ACT ATG GGG TCA GC
	Primer rev (5'-3')	CTC TGT GCA TTC TTG CTT CG
	SYBR Green	

are normally distributed, One-way ANOVA LSD post hoc test was used to define if there is any difference among the four groups. For data which are non-normally distributed, Mann–Whitney *U* test with Bonferroni correction was used to define if there is any difference between two groups.

RESULTS

PELLET CULTURE

Biochemical analysis. No differences were seen in DNA content between any of the groups (data not shown). Total GAG synthesized (including that released into the medium during the 8 days of culture) was normalized to the respective DNA content (Fig. 1A). Statistics were performed on the total GAG/DNA value (pellet + medium). Addition of 1 ng/ml of TGF-β1 increased the GAG synthesis by 28% compared with control, while 1 μM of LE135 decreased the GAG synthesis by 33%. Pellets cultured with both TGF-β1 and LE135 had a GAG/DNA value comparable to unstimulated control.

Gene expression. The COL1, COL2, COL10, AGG, Sp7, TGFB1, TGFB3, Sox9, and PTHrP gene expression levels of pellets cultured for 8 days were compared with the expression levels of cells on day 0 of culture (after trypsinisation and before pellet culture) (Fig. 2). Pellets cultured in the presence of 1 ng/ml of TGF-β1 showed

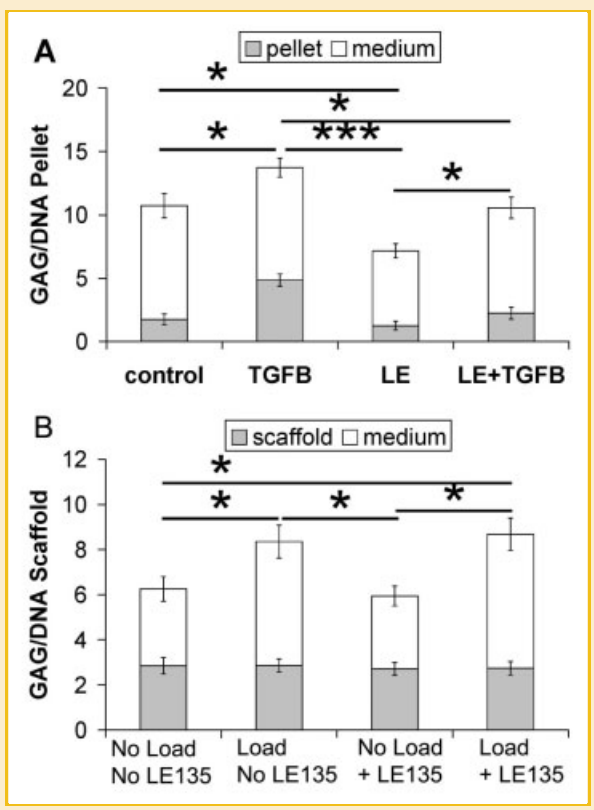

Fig. 1. GAG/DNA value of pellets and scaffolds cultured for 8 days, and their conditioned medium. Mean ± S.E.M., n = 12. $^{*}P < 0.05$, $^{***}P < 0.001$. Statistics were performed on the total GAG/DNA value (pellet + medium, scaffold + medium).

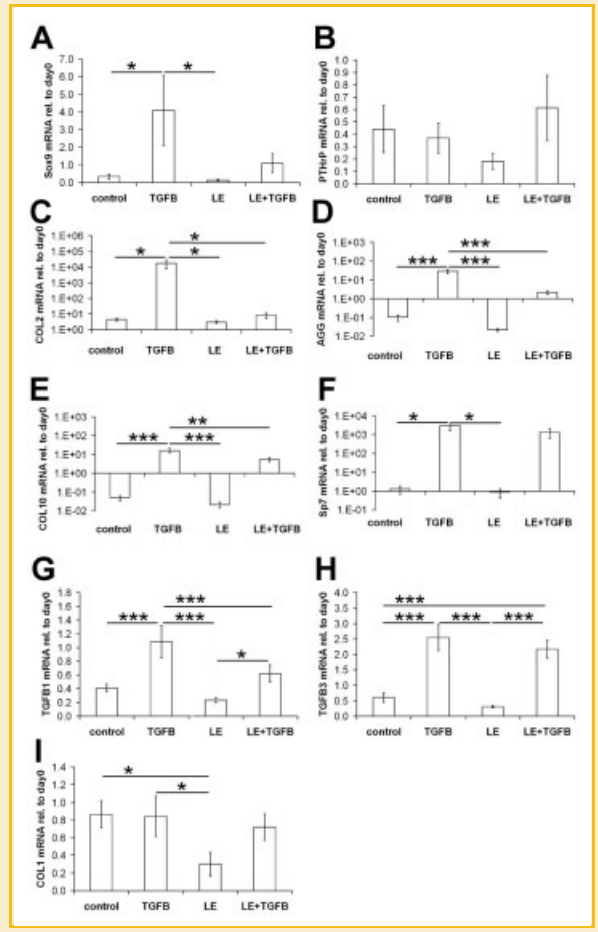

Fig. 2. Relative mRNA expression of hMSCs pellets cultured for 8 days with or without 1 ng/ml TGF-β1, and with or without 1 μM LE135 in the culture medium. Genes involving chondrogenesis (Sox9, Col II, Agg, PTHrP), hypertrophy (Col X), osteogenesis (SP7, Col I), and signal pathways (TGF-β1 and -β3) were investigated. Data were normalized to the expression level of cells before starting pellet culture (day 0). Mean ± S.E.M., n = 9. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

significantly higher gene expression of COL2 (3970-fold, $P < 0.05$), AGG (285-fold, $P < 0.001$), COL10 (325-fold, $P < 0.001$), Sp7 (2402-fold, $P < 0.05$), TGFB1 (2.64-fold, $P < 0.001$), TGFB3 (4.22-fold, $P < 0.001$), and Sox9 (12-fold, $P < 0.05$) compared with the control (Fig. 2, TGFB vs. control). In the pellets cultured with TGF-β1, addition of LE135 inhibited the increased gene expression of COL2, AGG, COL10, Sp7, and Sox9 seen in the TGFβ alone group. However, TGFB1 and TGFB3 gene expression was still up-regulated by addition of exogenous TGF-β1 to the culture medium (Fig. 2, LE + TGFB vs. LE). The inhibitor LE135 down-regulated the mRNA expression of COL1 by 65% ($P < 0.05$) compared with control (Fig. 2, LE vs. control). Addition of LE135 to TGF-β1 cultured pellets down-regulated the gene expression of COL2 ($P < 0.05$), AGG ($P < 0.001$), COL10 ($P < 0.01$), and TGFB1 ($P < 0.001$) compared to pellet group TGFB. However, addition of LE135 to TGF-β1 cultured pellets did not affect the Sp7 gene expression compared to pellet group TGFB (Fig. 2, LE + TGFB vs. TGFB). Gene expression of PTHrP did not show any difference among the four groups (Fig. 2).

SCAFFOLD CULTURE

Biochemical analysis. No differences were seen in DNA content between any of the four groups (data not shown). Total GAG synthesized (including that released into the medium during the 8 days of culture) was normalized to the respective DNA content (Fig. 1B). Statistics were performed on the total GAG/DNA value (scaffold + medium). In both groups where scaffolds were cultured with or without LE135, mechanical load enhanced the total GAG synthesis compared with the non-loaded control samples, indicating that LE135 did not inhibit GAG synthesis as seen in the pellet culture system. The increased amount of GAG was released to the medium, instead of being retained in the scaffold. This is because the pericellular matrix is not sufficiently developed to retain the entire GAG produced, which combined with the extremely porous nature of the scaffold/fibrin composite, results in a large proportion of the newly synthesized matrix being released. However, in the context of present tissue engineering work where the initial cells used had no GAG content at all, any GAG detected anywhere in the system must be newly synthesized.

Gene expression. The Sox9, AP1/Jun, COL2, COL10, AGG, Sp7, TGFB1, TGFB3, COL1, and PTHrP gene expression levels of scaffolds cultured for 8 days were compared with the expression levels of cells on day 0 of culture (after trypsinisation and before seeding into the scaffolds) (Fig. 3).

In the no LE135 group, mechanical load enhanced the expression of COL2 (70-fold, $P < 0.001$), AGG (16-fold, $P < 0.05$), COL10 (seven-fold, $P < 0.001$), Sp7 (75-fold, $P < 0.01$), Sox9 (1.58-fold, $P < 0.05$) and PTHrP (five-fold, $P < 0.001$) compared with respective control samples (Fig. 3, no LE135 load vs. no LE135 control). Addition of LE135 to the unloaded control samples had no effect on any gene above (Fig. 3, +LE135 control vs. no LE135 control).

In the group containing LE135, mechanical load did not enhance gene expression of COL2 or Sox9 as seen in the loaded group which did not have LE135. Addition of load, in combination with LE135, down-regulated AP1 expression ($P < 0.05$, Fig. 3). When LE135 was added to the culture medium, AGG gene expression was only slightly up-regulated by mechanical load (six-fold, $P < 0.05$, Fig. 3). However, the COL10 (eight-fold, $P < 0.001$), Sp7 (39-fold, $P < 0.001$), TGFB3 (two-fold, $P < 0.05$), and PTHrP (six-fold, $P < 0.001$) fold increase from control to loaded samples was not diminished by LE135 (Fig. 3, +LE135 load vs. +LE135 no load).

Addition of LE135 to the loaded scaffolds down-regulated COL2 gene expression ($P < 0.01$) while increased PTHrP gene expression ($P < 0.05$) (Fig. 3, + LE135 load vs. no LE135 load).

The expression of RARβ was also investigated and it could be detected in all groups. Both treatments applied affected the expression of this receptor. In the presence or absence of LE135, mechanical load down regulated RARβ expression. However, the application of LE135 increased mRNA expression under loaded and free swelling conditions (Fig. 4).

TGF-β1 ELISA. The concentration of TGF-β1 protein in the conditioned medium on days 2–8 (7 days of loading) was determined by ELISA. TGF-β1 protein concentration was higher in the loaded samples (0.48 ± 0.06 ng/ml) compared with the control samples (0.36 ± 0.04 ng/ml) when LE135 was not added (Fig. 5, no LE135 load vs. no LE135 control). Addition of LE135 to both control and loaded samples did not affect the TGF-β1 protein concentration in the culture medium (Fig. 5, +LE135 control vs. no LE135 control, +LE135 load vs. no LE135 load). In the LE135 added group, mechanical load also increased TGF-β1 synthesis. However, this increase was not significant (Fig. 5, +LE135 load vs. +LE135 no load).

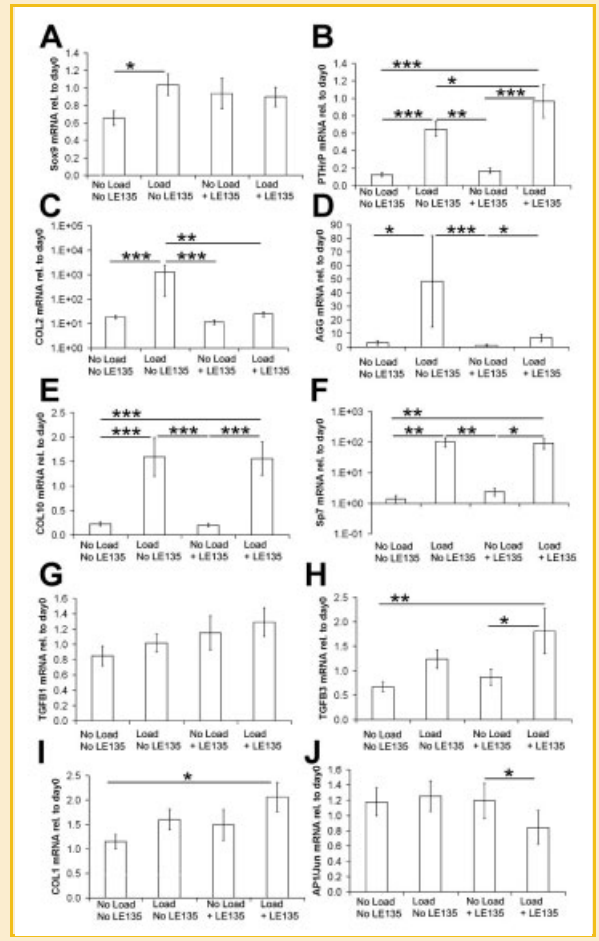

Fig. 3. Relative mRNA expression of hMSCs scaffolds cultured for 8 days with (load) or without (no load) mechanical load, and with or without 1 μM LE135 in the culture medium on days 2–8. Genes involving chondrogenesis (Sox9, Col II, Agg, PTHrP), hypertrophy (Col X), osteogenesis (SP7, Col I), and signal pathways (TGF-β1, -β3, and AP1) were investigated. Data were normalized to the expression level of cells before starting scaffold culture (day 0). Mean ± S.E.M., n = 12. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DISCUSSION

We investigated changes in the early gene expression profiles of human MSCs using two different 3D culture systems (pellet and a polyurethane/fibrin composite) during chondrogenic induction. In the classical pellet culture chondrogenesis was induced with addition of exogenous TGF-β, while in the scaffold system chondrogenesis was induced by way of mechanical load resulting in endogenous TGF-β synthesis [Li et al., 2010a]. Our previous results have shown that load induces chondrogenesis by increasing

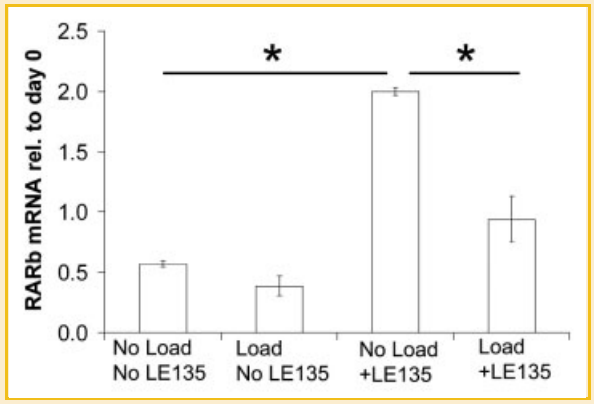

Fig. 4. Relative mRNA expression of RARβ in hMSCs scaffolds cultured for 8 days with (load) or without (no load) mechanical load, and with or without 1 μM LE135 in the culture medium on days 2–8. Data were normalized to the expression level of cells before starting scaffold culture (day 0). Mean ± S.E.M., n = 9. $^{*}P < 0.05$.

endogenous TGF-β synthesis [Li et al., 2010a] and the total GAG synthesized is proportional to the TGF-β concentration in the medium [Kupcsik et al., 2010].

Within this study, we used only one concentration of LE135, 1 μM. For consistency, this is the same concentration of LE135 used in previous studies [Kafienah et al., 2007]. The results presented here show that LE135 itself did not induce any increases in chondrogenic gene expression in either pellet or scaffold culture of human bone marrow MSCs. The only benefit of using LE135 is the down-regulation of type I collagen in pellet culture (Fig. 2, +LE135 vs. no LE135 control). However, this was not observed in scaffold culture (Fig. 3, +LE135 vs. no LE135 control). This may seem to be in contrast with the conclusion of Kafienah's study. However, the two studies are not directly comparable due to the difference of 2D and 3D culture. We observed type II collagen and aggrecan gene expression increase of 3970-fold and 285-fold, respectively, after 8 days of culture with 1 ng/ml of TGF-β1 in the pellets (Fig. 2, TGFB vs. control). In Kafienah's study in 2D culture, the type II collagen

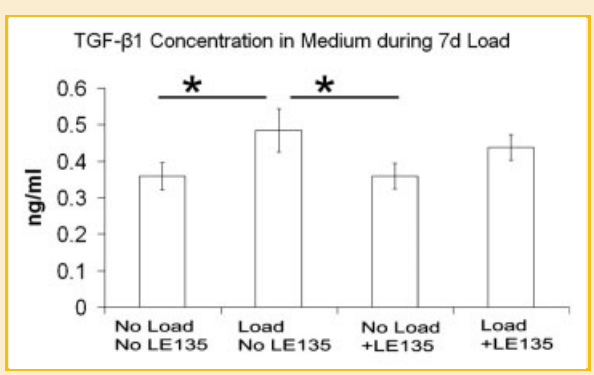

Fig. 5. TGF-β1 protein concentrations in the conditioned medium during 7 days of mechanical load. Scaffolds were cultured with (load) or without (no load) mechanical load, and with (+LE135) or without (no LE135) 1 μM LE135 on days 2–8. Mean ± S.E.M., n = 12. $^{*}P < 0.05$.

and aggrecan gene expression only increased three-fold each over the negative control after 6 days of monolayer culture with 10 ng/ml of TGF-β3, the LE135 treated group had a comparable gene expression level. Using safranin O stained sections of 3D polyglycolic acid (PGA) scaffolds cultured for 3 weeks, the same study previously showed that TGF-β3 treatment led to much more extensive proteoglycan stain than LE135 treated scaffolds [Kafienah et al., 2007]. This suggests that the effects of LE135 are dependant on the specific culture system used, although a weakness of the current study is the lack of 2D controls to directly compare to Kafienahs results. Also, as the 3D culture in PGA scaffolds did not have a control group lacking TGFβ (nor a combined LE135/TGFβ group) a direct comparison between the two studies is not possible. While both the study of Kafienah and the one currently presented were carried out in the absence of serum, it is important to note that many studies involving RA involve serum containing medium and this will undoubtedly have an influence on the results obtained. It is also interesting to note that both studies could detect changes caused by LE135 and yet the serum free conditions used do not contain any source of retinoic acid (or Retinol). Mammalian cells are incapable of synthesizing retinoids *de novo*, so in the absence of serum, the RARs should be unliganded and functioning in a repressive manner. One plausible explanation is that it is known cells have a high endogenous concentration of retinyl esters and this pool has a half-life of 24–48 h [Eppinger et al., 1993; Zouboulis et al., 1999]. During the proliferation stage, the cells are in 10% FCS where stores can be maintained, these would then be gradually used during the early stages of serum free culture. If this is the case, it would suggest that the early inhibition causes early fate decisions which are maintained. This would require further work to be carried out to confirm.

Addition of 1 μM LE135 to TGF-β treated pellets decreased the total GAG/DNA value (Fig. 1A, +LE135 + TGFB vs. no LE135 + TGFB), type II collagen, aggrecan, type X collagen, and TGFB1 gene expression (Fig. 2, +LE135 + TGFB vs. no LE135 + TGFB). In scaffold culture, addition of LE135 to loaded samples down-regulated type II collagen and aggrecan gene expression (Fig. 3, +LE135 load vs. no LE135 load).

LE135 had little effect on collagen X expression. In pellet culture there was a statistically significant decrease in collagen X expression compared to control, however the absolute change was small, raising questions to its biological significance. In scaffold culture no difference in collagen X expression was seen. A number of groups have questioned the validity of collagen X as a marker for hypertrophy due to the fact it is often seen within a few days of chondrogenic induction, sometimes even earlier than collagen II [Mwale et al., 2006]. Within our system the upregulation of collagen X during chondrogenic induction in the presence of LE135, while aggrecan, collagen II, and Sox9 are not upregulated, also raises questions as to the significance of the collagen X increase. These data indicate that LE135 actually inhibited the chondrogenic response induced by exogenous or endogenous TGF-β. However, the TGF-β gene expression and protein synthesis was not completely blocked with LE135, which suggests that the interference occurred downstream of TGF-β signaling. It has been shown that LE135 strongly represses AP-1 (Jun-Fos) activity in both HeLa and breast

cancer cells when RARβ was expressed [Li et al., 1999]. An important property of TGF-β1 is its ability to activate its own mRNA expression and thereby increase its own secretion. Both components of AP-1 complex are required for TGF-β1 auto-induction in human lung adenocarcinoma cells [Kim et al., 1990]. We observed a down-regulation of AP1 mRNA message when adding LE135 to the loaded samples. It could be that in our study LE135 diminished AP-1 activity, hence suppressing the auto-induction of TGF-β1 and the chondrogenic phenotype change induced by TGF-β1. Further studies would be required to confirm this hypothesis. Alternatively, LE135 is also a inhibitor of retinoic acid receptor α (RARα), although it is six-fold more selective for the β over the α receptor [Li et al., 1999]. One other study has used it as a RARα antagonist [Bai et al., 2009]. Kafienah et al. showed an increase in RARα mRNA expression after differentiation of MSCs to chondrocytes (3 weeks 3D culture) [Kafienah et al., 2007]. It might be that the inhibition of RARα by LE135 depressed the chondrogenesis effect.

Although LE135 inhibited any increase in chondrogenic gene expression induced by TGF-β, the expression of genes normally associated with osteogenesis was not affected. Retinoic acid (RA) has been shown to promote the differentiation of primary osteoblasts in vitro, resulting in enhanced expression of osteogenic genes and ultimate bone nodule deposition [Song et al., 2005]. Another study has determined that RA is required for osteogenic differentiation of mouse adipose-derived adult stromal cells [Wan et al., 2006]. However, in the present study, the inhibitor of RARβ did not influence the osteogenic gene expression in hMSCs. This may be due to species difference or due to the short term nature of the experiments described here. Alternatively, it indicates that some other retinoic acid receptor isoform is responsible for osteogenic differentiation. It has been shown recently that under serum free conditions RA and TGF-β pathways interact by regulation of TG-interacting factor (TGIF) during chondrogenesis of mouse embryonic palate mesenchymal cells [Zhang et al., 2009]. In this study RA inhibited TGF-β3 chondrogenesis and so it would be expected that blocking the RA receptor would promote chondrogenesis, or at the least not inhibit. As our study has the opposite conclusion it is possible that the different isoforms of RAR play different roles in the differentiation fate decision. Our data suggest that the inhibitor LE135 affects the osteochondral differentiation pathway at a different stage, permitting osteogenic gene expression while inhibiting chondrogenic gene expression. Alternatively, it might be that different cells were proceeding down different lineages. Some cells were undergoing chondrogenesis and this was affected by LE135, while other cells underwent osteogenic differentiation and were not affected by LE135.

Interestingly, PTHrP gene expression was not affected by exogenous TGF-β1 in pellet culture, but was upregulated by mechanical load in scaffold culture. This would indicate that its regulation is different to the large number of chondrogenic genes up-regulated by TGF-β exposure. The upregulation of PTHrP by load was not diminished by LE135.

In summary our data would indicate that under the two different culture conditions investigated application of the RARβ inhibitor LE135 inhibited chondrogenic gene expression induced both by exogenous TGF-β application and mechanical load. No effect was seen on oesteogenic gene expression under the same conditions.

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