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Gene expression by marrow stromal cells in a porous collagen-glycosaminoglycan scaffold is affected by pore size and mechanical stimulation

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Abstract Marrow stromal cell (MSC) populations, which are a potential source of undifferentiated mesenchymal cells, and culture scaffolds that mimic natural extracellular matrix are attractive options for orthopaedic tissue engineering. A type I collagen-glycosaminoglycan (CG) scaffold that has previously been used clinically for skin regeneration was recently shown to support expression of bone-associated proteins and mineralisation by MSCs cultured in the presence of osteogenic supplements. Here we follow RNA markers of osteogenic differentiation in this scaffold. We demonstrate that transcripts of the late stage markers bone sialoprotein and osteocalcin are present at higher levels in scaffold constructs than in two-dimensional culture, and that considerable gene induction can occur in this scaffold even in the absence of soluble osteogenic supplements. We also find that bone-related gene expression is affected by pore size, mechanical constraint, and uniaxial cyclic strain of the CG scaffold. The data presented here further establish the CG scaffold as a potentially valuable substrate for orthopaedic tissue engineering and for research on the mechanical interactions between cells and their environment, and suggest that a more freely-contracting scaffold with larger pore size may provide an environment more conducive to osteogenesis than constrained scaffolds with smaller pore sizes.

Introduction

A highly porous tissue engineering scaffold made from a copolymer of type I collagen (Col I) and glycosaminoglycan (CG scaffold) has been used clinically in skin regeneration [1]. Fabrication of this scaffold has recently been improved to give a relatively homogenous pore size and shape [2], and average pore size can also be varied over a wide range [1, 3]. It is known that pore size can have significant effects on the behaviour of cells migrating or seeded into scaffolds, varying with cell type [1, 3-6]. It is hoped that it may be possible to optimise the CG scaffold for a variety of applications, including orthopaedic tissue engineering. The scaffold has recently been shown to support expression of Col I, an early marker of osteogenesis, deposition of calcium phosphate and production of osteocalcin (OCN), a late marker associated with mineralisation, by marrow stromal cells (MSCs) cultured in the presence of osteogenic supplements [7].

The CG scaffold can be easily deformed when hydrated. This property has been utilised to investigate cell-mediated scaffold contraction, e.g. by fibroblasts [8, 9], chondrocytes [10], tendon cells [11] and an osteoblastic cell line [12]. It would also allow constructs to be subjected to cyclic extension or compression in a three-dimensional (3D) setting that mimics aspects of the physiological environment. A variety of effects have been reported for the application of mechanical stimulation, including cyclic strain, to cells cultured on two-dimensional (2D) and 3D substrates, and these effects may depend on cell and substrate type and the nature of the mechanical loading. In the mesenchymal lineage, including preparations enriched for stem cells [13],

low magnitude substrate strain may induce aspects of the osteoblastic phenotype in 2D [14-16] and 3D [17] culture, as may fluid flow [18-20]. Modelling and data obtained *in vivo* [15] suggest that strains as high as 5% may be compatible with intramembraneous osteogenesis during fracture healing. Strain on individual cells in a 3D context is likely to be less than that experienced by the bulk tissue or cell-scaffold construct [21]. On stretching our seeded scaffolds, it is expected that individual cells would experience some combination of strain, compression and fluid flow, depending on their position and the geometry of their attachment to struts in various directions [22], and that pore size might also have an effect on the mechanical stimuli experienced by the cell population. We propose that it may be possible to derive suitable mechanical stimulation regimens to direct specific cell differentiation and matrix deposition within these scaffolds prior to implantation [23, 24].

The hypotheses tested here are that gene expression in MSCs differs between 2D plastic versus 3D CG scaffold culture and that it also depends on pore size and the degree of mechanical stimulation in the 3D constructs. To test these ideas, we measure levels of the RNAs for Col I and OCN, as well as for two additional bone-associated markers, osteopontin (OPN) and bone sialoprotein (BSP), in MSCs cultured under a variety of conditions. OPN can bind to cell surface receptors, other matrix proteins and calcium deposits, and is thought to be involved in cell adhesion, signalling and regulation of hydroxyapatite crystal formation [25], and overexpression promotes osteogenic functions in cultured cells [26]. BSP, like OCN, is a late marker expressed around the time of mineralisation. While standard antibody-based assays provide information about the accumulated or steady state levels of a protein in a tissue at the time of sampling,

measurements of gene expression at the RNA level provide complementary information, in the form of an insight into the capacity of the cells to make that protein at that time.

Materials and methods

CG scaffold

CG scaffolds (95.5% porosity) of average pore size 96, 110 and 151 μm were produced and characterised as described by O'Brien et al. [3].

Cell culture

Three experiments were performed with MSCs cultured as follows:

Expt. 1: Culture on plastic (2D) versus CG scaffold (3D), in control medium versus osteogenic medium;

Expt. 2: Culture in osteogenic medium in CG scaffolds of different pore size;

Expt. 3: Culture in osteogenic medium in CG scaffolds that were unconstrained, clamped in a rig, or clamped in a rig with intermittent cyclic strain.

Marrow was collected from the femoral and tibial bones of Wistar rats sacrificed by CO_2 asphyxiation. EU and Irish guidelines for the care and use of laboratory animals (SI 566/2002) have been observed. MSCs were isolated on the basis of plastic adherence, as described in reference 7: Marrow was flushed from bone shafts using a 25-gauge needle with culture medium consisting of Dulbecco's modified Eagle medium (DMEM, Sigma-

Aldrich D5671 formula) supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100 µg/ml streptomycin, 2 mM Glutamax, 1 mM L-glutamine and 1% non-essential amino acids. The marrow was pelleted for 5 min at 650 g, passed sequentially through 16-, 18-, and 20-gauge needles, then through a 40 µ cell strainer to remove clumps. This suspension was incubated in a 10 cm plastic Petri dish for a short time (30 min) to deplete white blood cells, and the supernatant was plated into two T75 tissue culture flasks. Cells that were non-adherent after 24 hours were later removed by rinsing and replacing medium in these flasks. Upon reaching 80–90% confluence, the cells were passaged and replated into two T175 flasks. Adherent proliferating cells were further passaged for up to three weeks (approx. passage four) to doubled flask numbers as they expanded, and half of the medium was replaced at least every 3-4 days if time between passages took longer. Different batches of MSCs were used for each experiment. All culture incubations were carried out at 37°C, 5% CO₂ and 95% relative humidity. Control medium for Expt. 1 was as for expansion, while osteogenic medium contained, in addition, 10 nM dexamethasone, 50 µM L-ascorbic acid 2-phosphate and 10 mM β-glycerophosphate.

For Expts. 1 and 2, pieces of CG scaffold (approximately 1 cm², 3 mm thick) were seeded in 6-well culture plates (well-bottoms coated with 2 ml of 2% agarose, one scaffold per well) with 5x10⁵ MSCs suspended in control medium. Half of the cells were applied to one side of the scaffold in 150 µl and incubated for 30 min. The scaffold was then turned over and the second half of the cells applied in 150 µl (Expt. 1) or 38 µl (Expt. 2) and incubated for a further 30 min. Medium (2 ml) was then added and cultures returned to the incubator. For 2D cultures, 5x10⁴ cells were applied per well (uncoated) in 2 ml of medium. After 2 days, initial samples were collected for analysis, and liquid

surrounding the remaining cultures was then replaced with osteogenic or fresh control medium; these initial samples are designated “day zero” with respect to the time of initiating osteogenic treatment. When samples were harvested for analysis at subsequent time points, half of the liquid in each of the remaining cultures wells was replaced with fresh medium, and this was repeated at additional times as needed to maintain a twice-weekly feeding schedule.

In order to generate an external standard RNA stock with high levels of bone-associated transcripts, rat osteosarcoma cell line UMR106 (obtained from ATCC) was cultured on plastic to confluence in 30-2002 formula DMEM from ATCC supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and then for an additional six days after further supplementing the medium with 50 µg/ml L-ascorbic acid 2-phosphate, replacing medium every second day.

Stretching MSC-seeded scaffolds

For the uniaxial strain experiment (Expt. 3), rectangular pieces of scaffold (1 cm x 3 cm, 3 mm thick; 96 µm pore size) were clamped in mechanical rigs [22] as follows (see Fig. 1): In order to prevent sagging when wetted, scaffolds was supported by similar-sized pieces of silicone sheet, and 0.5 cm of the scaffold was attached to the silicone at each end using liquid polydimethylsiloxane as a glue. These were left at room temperature to set overnight. One silicone-glued terminus of each scaffold was attached in a screw-down clamp to one end of a stretching frame, and then the other end was similarly immobilised, with the central 2 cm of the scaffold held straight, but under zero strain, between the clamps. Frames were placed

into purpose-designed sterile steel receptacles for culture. Clamped scaffolds were seeded on their upper sides only (1 cm x 2 cm exposed area) by applying 10^6 MSCs in 400 μ l of medium. Unconstrained control scaffold sections of the same size were seeded on one side in agarose-coated culture dishes. Constructs were incubated for 60 min before adding medium to just cover the frame-mounted scaffolds (18 ml) and to give the same ratio of medium to cells around the unconstrained constructs. After 2 days, the control medium was replaced with osteogenic medium, and half replaced after a further 4 days (“day 4” of osteogenic culture). The following day (day 5), half of the frame-mounted constructs were connected to a stretching device and subjected to 5% cyclic strain at 1 Hz for 4 hours. This stretching regimen was repeated on the following two days (days 6 and 7) before harvesting all constructs for RNA extraction. We used MSC batches in which levels of BSP and OCN RNAs at this time point reached at least 0.5% of the external calibrator used for Expts. 1 and 2.

Quantitation of RNAs from specific genes

Total RNA was extracted using the RNeasy[®] Mini Kit and RNase-Free DNase Set (Qiagen) according to the manufacturers’ instructions; scaffolds were disrupted in 600 μ l of kit lysis buffer using an Omni electric homogeniser. Target RNAs were quantified by reverse transcription followed by real-time PCR, as follows: Reverse transcriptions (10 μ l) were performed on 100 ng of RNA by random hexamer priming using TaqMan[®] Reverse Transcription Reagents (Applied Biosystems), with 12.5 U of reverse transcriptase at 48°C for 30 min. Real-time PCR reactions (10 μ l) were performed in duplicate (Fig. 2 samples),

triplicate (Fig. 1 samples) or quintuplicate (Fig. 3 samples) on 0.5 (18S rRNA), 1 (collagen I $\alpha 2$ (Col I), OPN) or 10 ng (BSP, OCN) cDNA, using TaqMan[®] Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, with primers at 900 μ M, except for 18S rRNA (300 μ M), and 5'-Fam-/3'-Tamra-labelled probes at 250 μ M; primers and probes (MWG) were designed using Primer Express software (Applied Biosystems) and are listed in Table 1. Relative levels of RNAs from MSCs cultured under different conditions were calculated using standard curves of Ct versus log of input RNA quantity as described in Applied Biosystems User Bulletin No. 2. These standard curves were generated from a series of ten-fold dilutions of RNA extracted from the differentiated UMR106 culture described above. Col I, OPN, BSP and OCN values were then normalised to 18S rRNA as endogenous reference. For Expts. 1 and 2, values were expressed relative to the UMR106 levels (i.e. Col I in these cells is set as 100% etc.), while the calibrator for Expt. 3 is the average unconstrained culture value.

Statistical analysis

Statistical analysis was carried out using one-way or two-way analysis of variance (ANOVA) as noted in text and figure legends followed by the post-hoc Bonferroni's post tests when significance ($P < 0.05$) was indicated.

Results

Time course of osteodifferentiation of MSCs cultured in the CG scaffold (3D) versus plastic (2D)

In order to investigate the behaviour of rat MSCs in the type I collagen-GAG scaffold, we measured the expression of four bone-associated genes up to 21 days in culture, with and without osteogenic supplements, and compared these to similar cultures on standard tissue culture plastic. Figure 2 shows one representative of five runs of the experiment, each performed with MSCs from a different rat. Between runs, there was a high degree of variability (not shown) in the extent to which the bone-associated RNAs, especially for BSP and OCN, were expressed and upregulated. Because of this variability, statistical significance could not be established for the differences in outcome between the culture conditions of interest; however, when there was meaningful expression of the late markers, BSP and OCN, levels of these RNAs were always greater in the scaffold than on plastic.

The Col I RNA levels displayed the least modulation of expression (Fig. 2A), and are comparable to some of the highest that we observed during culture of the rat osteosarcoma cell line UMR106 (Fig. 2 charts are calibrated relative to RNA from these cells). Osteopontin expression did not appear to be reproducibly upregulated in the scaffold during culture, although there was clear induction in the 2D culture with osteogenic supplements (Fig. 2B), to levels comparable with those reached by UMR106 cells.

In contrast, the CG scaffold was associated with marked induction of the mineralisation-associated genes BSP and OCN, even in the absence of osteogenic factors;

this upregulation exceeded that seen in 2D, where osteogenic factors were required (Figs. 2C and 2D). The presence of osteogenic supplements in the 3D cultures caused earlier, rather than greater, increases in BSP and OCN expression in the experiment shown here.

MSC osteodifferentiation in scaffolds having larger and smaller pore sizes

The average pore size of the scaffold used in the experiment of Fig. 1 is 110 μm . We compared the levels of bone-associated RNAs from MSCs cultured in osteogenic medium within a scaffold of this type with levels from cells cultured in variants having average pore sizes of 96 and 151 μm . Pore size had significant effects on expression of Col I ($p < 0.05$), OPN and OCN ($p < 0.001$, 2-way Anova; Fig. 3), with larger pore environments tending to accelerate induction or increase levels of these RNAs. Pore size effects do not become apparent until the third sampling point (day 14) and showed significant overall variation with time of culture for OPN ($p < 0.001$) and OCN ($p < 0.01$). Specifically, the middle and possibly largest (just below significance) pore sizes favour Col I expression at day 14, with only the largest having an advantage at day 21; OPN increases through the pore sizes at day 14, but expression levels out at day 21; and the largest pore size favours OCN expression at both these time points.

Application of mechanical constraint and uniaxial cyclic strain to MSC-seeded scaffolds in osteogenic medium

To examine the effects of mechanical stimulation on osteodifferentiation in a 3D environment, MSC-seeded CG scaffolds were exposed to intermittent cyclic strain. Day 7 of culture in osteogenic medium was chosen for endpoint analysis, since at this stage the initiation of a definite osteogenic response can be seen at the RNA level for BSP and OCN (Fig. 2C and D). The constructs were immobilised in the starting position between stretching sessions, and were thus both constrained and intermittently stretched compared to the standard conditions of culture for the materials presented in Figs. 2 and 3.

Therefore, two sets of control/comparison constructs were cultured in parallel: (i) unconstrained scaffolds corresponding to the standard conditions but with seeding details and media volumes as for the stretched constructs, and (ii) scaffolds which were prevented from contracting, as for the experimental constructs, by clamping in a rig, but which were not stretched. Fig. 4E illustrates contraction of an unconstrained scaffold.

The mechanical conditions had significant effects on the levels of OPN, BSP and OCN RNAs. These RNAs were reduced in clamped relative to unconstrained scaffold culture (Fig. 4 B-D). In addition, there was a small but significant increase in OPN RNA associated with stretching the clamped constructs (Fig. 4B), i.e. a response in the opposite direction to that observed for constraint.

Discussion and conclusions

Here we tested the hypotheses that the expression of genes associated with osteogenic differentiation of marrow stromal cells would be affected by culture in 3D on a type I collagen-GAG scaffold, compared to 2D culture on plastic, and by the pore size and

mechanical manipulation of this scaffold. We found evidence to support each of these hypotheses for at least some of the genes we examined.

The highest BSP and OCN RNA levels that we see in the scaffold cultures are comparable to or greater than those that we have observed developing in UMR106 cells (Fig. 2C and D). Although measuring RNAs in cell populations does not provide information about the levels reached in individual cells, this suggests that a large proportion of the MSCs may be differentiating to osteoblasts in the CG scaffold. The data also suggest that the CG scaffold is enhancing the osteogenic response over that observed in 2D culture in the presence of soluble osteogenic supplements, and show that robust osteodifferentiation can occur on the scaffold even in the absence of such supplements.

We do however note some apparent differences from the results of previous studies of OCN at the protein level by immunofluorescent staining. Firstly, Farrell et al. [7, 27] did not detect the protein in CG scaffolds cultured in the absence of osteogenic supplements. Although the level of OCN RNA expression we observe without osteogenic supplements in the current study is on average lower in 2D than in the scaffold across the multiple runs of the experiment, it can reach high levels by day 21, as seen in Fig. 1D, and its protein product would be expected to be detectable on some occasions. Secondly, OCN protein was detected in 2D culture by immunofluorescent staining possibly earlier (day 7) than in parallel CG scaffold culture [27]. Quantification of both RNA and protein in samples from the same experiments would be required to determine whether there is a real discordance that would suggest that OCN RNA “induced” by the scaffold is not efficiently translated.

One osteogenic supplement, ascorbic acid, is necessary for processing collagen before secretion and fibre formation; deposition of an extracellular collagen matrix is normally an important part of the osteodifferentiation process, as the fibres interact with cell receptors (integrins), which initiate signalling to stimulate transcription of bone-related genes [28]. There are reports that some preparations of exogenous collagen I can stimulate aspects of the osteoblastic phenotype, probably via the natural collagen-integrin interaction [29, 30]. The 3D aspect of the CG scaffold as a cell substrate may also have an important role: Jaworski et al. [31] have recently demonstrated this to be true for lung fibroblasts seeded in a similar CG scaffold.

The lack of upregulation of Col I RNA by osteogenic factors in our experiment (Fig. 2A) was somewhat unexpected, as Farrell et al. [7] found that detection of the protein via immunohistochemistry in MSCs required osteogenic supplements. However, it is possible that the Col I RNA present in the absence of osteogenic supplements is not translated – there is evidence for modulation of collagen expression at the level of translation in other systems [32, 33]. Also, there are other reports of expression of Col I in undifferentiated MSCs [34]. We also note the relative lack of OPN upregulation in the scaffold compared to plastic culture (Fig. 2B), at the assayed time points; the significance of this observation is not yet known, although it may be noteworthy that this gene tends to be expressed earlier in osteogenesis than BSP and OCN.

With regard to the variability in gene expression observed for different MSC preparations in our study, there are many examples in the literature documenting related variability in marker expression between colonies derived from individual

osteoprogenitor cells [35, 36], between mature osteoblasts [37], between populations of human MSCs[38], and even, as here, populations of MSCs from inbred rat strains[39].

Our comparisons of MSC osteodifferentiation in three pore-size variants of the CG scaffold (Fig. 3) suggest that within the range examined here – 96 to 151 μm – pore size may have some influence on gene expression, with larger pore environments tending to accelerate induction or increase levels of the bone-associated RNAs. There are a number of parameters associated with increasing pore size that might affect cell behaviour, including a decrease in strut surface area per unit volume to which cells can attach [3] and an increase in unseeded scaffold permeability[40]. Pore size effects for this scaffold may well be cell-type dependent, as Nehrer et al. [5] found that chondrocytes synthesised more GAG in a very small (20 μm) pore CG scaffold than a 85 μm variant.

The mechanical constraint/stimulation experiment involved only one of many possible combinations of constraint, strain, scaffold and culture parameters. However, the results demonstrate that expression of at least three of the four bone-associated genes is sensitive to scaffold constraint, even in one direction: OPN, BSP and OCN RNAs are reduced in clamped relative to unconstrained scaffold culture (Fig. 4 B-D). Cells within our clamped scaffolds presumably experience extra resistance to the forces they exert that would contract the scaffold in the direction of the constraint, and their average proximity to scaffold struts and each other would be reduced compared to cells in unconstrained constructs – either or both of these differences might be involved in impeding differentiation. Other researchers have noted that cells, including dermal fibroblasts and osteoblasts, develop phenotypes consistent with more mature tissue in freely-contracting compared to constrained collagen gels [41-43]. The clamping data suggest that a more

freely-contracting CG scaffold may provide an environment more conducive to osteogenesis.

There was a small but significant increase in OPN RNA associated with stretching the clamped constructs (Fig. 4B), i.e. a response in the opposite direction to that observed for constraint. Other groups have found that OPN can be upregulated by cyclic strain, intermittent hydrostatic compression and oscillatory fluid flow in osteoblast lineage cells cultured in 2D [16, 18, 20]. In addition, the protein itself may have a role in facilitating cell attachment to the matrix [25] and contribute to mechanically-induced signalling responses [44]. Consistent with this scenario, cells with higher basal OPN RNA levels have been found to show higher upregulation of expression by intermittent hydrostatic compression [18]. If our MSCs behave similarly, allowing the seeded CG constructs to first undergo contraction, or freeing them from constraint between stretching sessions might increase the strain response.

In conclusion, the data presented here further establish the CG scaffold as a potentially valuable substrate for orthopaedic tissue engineering: they suggest that this scaffold promotes osteogenic differentiation of rat MSCs *in vitro*, and that pore size and mechanical stimulation can be optimised to control the process. The results suggest that a more freely-contracting scaffold may facilitate tissue development. Our findings may have parallels with those of Vickers et al. [43] who recently reported that contraction of type II collagen-GAG scaffolds by chondrocytes helps support chondrogenic activity. Like us, they propose a role for seeded scaffold contraction in stimulating mature tissue development, although the influence of cell density is likely to be even more important in the case of chondrogenesis. Recent surgical developments to repair osteochondral

defects include fixing freshly-seeded collagen scaffolds to the host tissue [45]. Our results and related observations by others advise general caution in such situations, as constraint may hamper development of mature tissue. Other groups may confine themselves to use of cell-scaffold ratios that produce less contraction *in vitro*, e.g. in the case of experimental implants for tendon repair [46]. We suggest further investigation of a role for free-floating pre-culture of constructs *in vitro*.

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FIGURE LEGENDS

[Figures should appear as close as possible to first mention in main text]

Fig. 1.

Schematic illustrating set-up of seeded scaffolds in stretching rig.

Fig. 2.

Relative levels of bone-associated RNAs for Col I (**A**), OPN (**B**), BSP (**C**) and OCN (**D**) from MSC-seeded plastic or CG scaffold substrates incubated for 0, 3, 7, 10, 14 or 21 days in the presence or absence of osteogenic factors; one representative experiment of five is shown. Day zero samples (striped bars) were taken two days after seeding, before osteogenic treatment. RNAs were quantitated by reverse transcription followed by real-time PCR. Levels were normalised to 18S rRNA as endogenous reference and expressed relative to an external calibrator RNA derived from rat osteosarcoma cell line UMR106 cultured in the presence of ascorbic acid. Some values in (**C**) and (**D**) are too small to be resolved on the charts.

Fig. 3.

Relative levels of bone-associated RNAs from MSC-seeded CG scaffold variants of 96, 110 or 150 μm average pore size. Constructs were sampled in triplicate† (n = 3) after 0, 3, 14 and 21 days of culture in osteogenic medium; (†duplicate for day 7 of the 96 μm scaffold). Normalised target RNA measurements and external calibrator as for Fig. 1. Bars show mean values \pm SD. Statistics: Two-way ANOVA with $*p < 0.05$, $**p <$

0.01, *** $p < 0.001$ indicated for Bonferroni's post tests. Part **E**: Summary of significant differences in gene expression between scaffolds of different pore size at each time point, where scaffold(s) to the left of the symbol “<” have a lower level of expression than scaffold(s) to the right.

Fig. 4.

Effects of one-dimensional clamping and 5% uniaxial cyclic strain (1 Hz) on levels of bone-associated RNAs from MSC-seeded CG scaffold constructs incubated for 7 days in the presence of osteogenic factors; constructs were unconstrained (Un), clamped (Cl), or clamped and stretched (Cl+St). Target RNA measurements were normalised to 18S rRNA as endogenous reference, then expressed as a percentage of the average value for the unconstrained samples for the respective MSC batch used, and charted as mean \pm SD for each condition; $n = 4$. Statistics: One-way ANOVA with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicated for Bonferroni's Multiple Comparison post test. Part **E**: Photograph of an unconstrained construct at time of harvest; the seeded side (upper) side has curled due to cell-mediated contraction of the scaffold.