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Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: Differential effects of 5-aza-deoxycytidine and trichostatin A

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ABSTRACT

Clinical imperatives for new bone to replace or restore the function of traumatized or bone lost as a consequence of age or disease has led to the need for therapies or procedures to generate bone for skeletal applications. However, current *in vitro* methods for the differentiation of human bone marrow stromal cells (HBMSCs) do not, to date, produce homogeneous cell populations of the osteogenic or chondrogenic lineages. As epigenetic modifiers are known to influence differentiation, we investigated the effects of the DNA demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) or the histone deacetylase inhibitor trichostatin A (TSA) on osteogenic and chondrogenic differentiation. Monolayer cultures of HBMSCs were treated for 3 days with the 5-aza-dC or TSA, followed by culture in the absence of modifiers. Cells were subsequently grown in pellet culture to determine matrix production. 5-aza-dC stimulated osteogenic differentiation as evidenced by enhanced alkaline phosphatase activity, increased Runx-2 expression in monolayer, and increased osteoid formation in 3D cell pellets. In pellets cultured in chondrogenic media, TSA enhanced cartilage matrix formation and chondrogenic structure. These findings indicate the potential of epigenetic modifiers, as agents, possibly in combination with other factors, to enhance the ability of HBMSCs to form functional bone or cartilage with significant therapeutic implications therein.

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1. Introduction

Tissue regeneration promises to deliver specifiable replacement tissues and the prospect of efficacious alternative therapies for orthopaedic applications such as non-union fractures, healing of critical sized segmental defects and regeneration of articular cartilage in degenerative joint diseases. A typical strategy is to isolate stem cells, derived from the patient, and to subsequently culture these under specific conditions that allow expansion of the stem cell population and subsequent differentiation to the desired cell type. Pre-differentiated or fully differentiated cells are implanted (usually in combination with an appropriate scaffold) in the patient providing replacement of the diseased, damaged or surgically excised tissue (Dawson and Oreffo, 2008). The current process of *in vitro* differentiation is, however, inefficient and the generation of homogeneous skeletal cell populations has, to date, proved elusive (Alexanian, 2007).

Epigenetic regulation of gene expression is recognised as a central mechanism that governs cell stemness, determination, commitment, and differentiation (Alexanian, 2007; Delcuve et al., 2009; Feinberg, 2007; Lotem and Sachs, 2006; Oreffo et al., 2005; Wu and Sun, 2006). Two important epigenetic mechanisms are associated with gene silencing, i.e. DNA methylation and histone deacetylation. DNA methylation takes place only on cytosine bases. 5-aza-deoxycytidine (5-aza-dC), an analogue of cytosine, cannot be methylated and inhibits DNMT1, the maintenance methyl transferase, thus preventing methylation of the nascent strand during replication (Haaf, 1995). Trichostatin A (TSA) inhibits histone deacetylases (HDACs) non-competitively and reversibly. Depending on the gene and cell type (Weidle and Grossmann, 2000; Yoshida et al., 1995), 5-aza-dC and TSA have been shown to enhance the differentiation of mouse BMSCs into neural-like cells (Alexanian, 2007) or mesenchymal differentiation from adipose cells (Boquest et al., 2006). Till date, the role of epigenetic modifiers on the modulation of skeletal cell differentiation remains far from clear. The aims of this

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study were to investigate whether epigenetic modifiers can influence the commitment of HBMSCs towards the osteogenic or chondrogenic lineage.

2. Materials and methods

2.1. Isolation of human bone marrow stromal cells

Human bone marrow stromal cells (HBMSCs) were obtained from six males (60–81 years) and one 92 year old female subject to total hip arthroplasty surgery. All samples were obtained with full consent and under approval of the local Regulatory Ethics Committee (LREC 194/99). HBMSCs Cells were separated and isolated using standard protocols and on average, each sample provided between 7×10^6 and 12×10^6 cells. The cell suspension was cultured in α-MEM, 10% FCS serum, 100 U/ml penicillin, and 100 U/ml streptomycin (p/s). Media were changed twice weekly. Only HBMSCs from passage 1 (P1) were utilized in all experiments.

2.2. Treatment regimen

HBMSCs were initially cultured under basal conditions in monolayer until 50% confluency was reached. Thereafter, serum was removed for 24 h, to synchronize cell division (Campisi et al., 1984; Pardee, 1989), as 5-aza-dC and TSA predominantly exert their effects during replication. 5-aza-dC (1 μM) or TSA (100 nM) were added to basal media containing 10% FCS on 3 successive days. Cultures were continued in either basal or osteogenic media (basal media with 100 μM ascorbate-2-phosphate and 10 nM dexamethasone). Cultures were harvested when the cells reached confluency (4–7 days after 50% confluency) and total RNA was extracted for gene expression analysis. In one set of experiments recombinant BMP-2 (100 ng/ml) was added to osteogenic media for comparison purpose (supplied by Prof. Walter Sebald, University of Würzburg, Germany).

2.3. Pellet culture

For pellet culture (Lee et al., 2001; Tare et al., 2005), 0.5×10^6 cells were transferred to 30 ml universal tube in 1 ml of media, centrifuged at 400 g for 10 min and then incubated at 37 °C, 5% CO_2 with loose caps to allow gas exchange. The cells formed a sphere within 24–48 h. Pellets were cultured either in osteogenic (α MEM with 100 U/ml p/s together with 10 μM dexamethasone and 100 μM ascorbate-2-phosphate) or chondrogenic conditions (α MEM with 100 U/ml p/s, 10 ng/ml TGF β3 (VWR), 10 μM dexamethasone, 100 μM ascorbate-2-phosphate and 1X insulin, transferrin and selenium (ITS). ITS supplements were used at the following concentrations: 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, and 0.5 μg/ml sodium selenite. Media were changed three times per week. Pellets were kept in culture for 21 days and fixed thereafter in 4% paraformaldehyde.

2.4. Histological methods

Pellets were fixed in 4% paraformaldehyde, embedded in wax, and sectioned at 6–7 μm. The following staining methods were used.

2.4.1. Alcian blue/Sirius red/Weigert's haematoxylin

Nuclei were stained with Weigert's haematoxylin (10 min) and dedifferentiated in 1% acid alcohol. Proteoglycans were stained with Alcian blue (0.5% in 1% acetic acid) for 10 min, followed by 1% molybdophosphoric acid (10 min), and Sirius red F3B (1% in 30% picric acid for 60 min). Sections were dehydrated and mounted in DPX.

2.4.2. Von Kossa

To demonstrate the formation mineralised matrix, sections were covered with 1% silver nitrate ($AgNO_3$) and placed under UV light for 20 min. After rinsing, sections were fixed with 2.5% sodium thiosulfate for 8 min, counterstained with van Giesen (180 mg Acid Fuchsin, 100 ml saturated picric acid, 100 ml H_2O), dehydrated and mounted with DPX.

2.4.3. Immunocytochemistry

Following quenching of endogenous peroxidase activity with 3% H_2O_2 and blocking with 1% bovine serum albumin (BSA) in PBS, sections were incubated with the relevant primary antiserum at 4 °C overnight, followed by incubation for an hour with the appropriate biotinylated secondary antibody.

The immune complex was visualised by the avidin–biotin method, linked to peroxidase and 3-amino-9-ethylcarbazole; this yields a reddish brown reaction product. Negative controls, in which the primary antibody was omitted, showed no staining. The polyclonal antibodies, Type I collagen (LF67 (Fleischmajer et al., 1990), a generous gift from Dr. Larry Fisher, NIH, Bethesda, USA; 1:300), Type II collagen (Calbiochem; 1:500), Sox-9 (Chemicon; 1:150) were raised in rabbits. The secondary antibody was an anti-rabbit IgG biotin-conjugated antibody (DAKO; 1:200). Pre-treatment with hyaluronidase type I-S from bovine testes (451 units/ml), used as 0.8 mg/ml of 1% BSA in PBS for 20 min at 37 °C, exposed the epitope of collagen II. Antigen retrieval was used for detection of Sox-9. In brief, slides were boiled in 0.01 M citrate buffer for 5 min.

2.5. Alkaline phosphatase (ALP) assays

Specific ALP activity was quantified using HBMSC monolayer cultures. The cell lysate was incubated for a recorded time with 100 μl of ALP buffer plus 1 mg/ml 4-nitrophenyl phosphate disodium salt hexahydrate at 37 °C. 4-Nitrophenol solution was titrated as a standard curve. The colour produced was read at the wavelength of 405 nm. The results were corrected for DNA content, which was quantified using the Picogreen dsDNA assay. Samples were compared to a standard curve by diluting herring sperm DNA with TE buffer. All samples were run in triplicate and fluorescence was measured at 485 nm (excitation) and 530 nm (emission). ALP enzyme specific activity was expressed as nmol PNPP/h/ng DNA.

2.6. Gene expression analysis

The all Prep DNA/RNA Mini Kit (Qiagen) was used to extract RNA and DNA from confluent monolayer cultures according to manufacturer's instructions. Reverse transcription was carried out immediately after RNA extraction, as previously described (Hashimoto et al., 2009) and the 'Applied Biosystems 7500' was used as Real time PCR system with Power SYBR® Green PCR Master Mix (Applera UK). PCR assays were performed in 25 μl using 12.5 μl of Power SYBR® Green PCR Master Mix, 2.5 μl each of forward and reverse primer (Runx2 (Abdallah et al., 2006) F: 5′ tct tca caa atc ctc ccc 3′, R: 5′ tgg att aaa agg act tgg tg 3′ and GAPDH F: 5′ cca ggt ggt ctc ctc tga ctt c 3′, R: 5′ tca tac cag gaa atg agc ttg aca 3′ final concentration was 500 nM for GAPDH and 1 μM for Runx2), 6.5 μl of ultrapure water and 1 μl of sample cDNA per reaction. Absence of primer dimmers was confirmed by dissociation curve/melt curve analysis. We also validated that the

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