Biochemical and Biophysical Research Communications 401 (2010) 20–25

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Bone morphogenetic protein 6 drives both osteogenesis and chondrogenesis in murine adipose-derived mesenchymal cells depending on culture conditions

Carly M. Kemmis, Ali Vahdati, Holly E. Weiss, Diane R. Wagner *

Department of Mechanical Engineering and Bioengineering Graduate Program, University of Notre Dame, Notre Dame, IN 46556, USA

ARTICLE INFO

Article history:
Received 19 August 2010
Available online 9 September 2010

Keywords:
Osteogenesis
Chondrogenesis
Adipose-derived mesenchymal stem cells
BMP-6
Culture conditions

ABSTRACT

Bone morphogenetic proteins (BMPs) play a dual role as a factor in both bone and cartilage development and correspondingly have the therapeutic potential to regenerate both tissues. Given this dual nature, previous *in vitro* research using BMPs has relied on distinct media formulations and culture conditions to drive undifferentiated cells to the osteogenic or chondrogenic lineage. To isolate the impact of culture conditions and to explore the effect of BMP-6 on murine adipose-derived mesenchymal cells (ASCs), ASCs were seeded in either monolayer or pellets in an identical medium containing BMP-6. Results indicate that BMP-6 differentially promotes osteogenesis and chondrogenesis in ASCs depending on culture conditions. BMP-6 potently induced alkaline phosphatase activity and mineralization in ASCs cultured in monolayer conditions. In contrast, BMP-6 enhanced proteoglycan accumulation in ASCs seeded in chondrogenic pellet culture. A comparison of gene expression suggests that the differentiating effect of BMP-6 is specific to the particular culture condition. This study highlights the importance of the interactions between chemical signaling and microenvironmental cues in directing cell fate.

1. Introduction

Bone morphogenetic proteins (BMPs) are signaling molecules in the transforming growth factor-beta (TGF-β) superfamily that play significant roles in both bone and cartilage morphogenesis [1] and are also effective in bone and cartilage repair [2–4]. BMP-2 and BMP-7 are clinically available to enhance and accelerate bone repair and formation (INFUSE®, Medtronic, Sofamor Danek, Memphis, TN; OP-1 Implant® and OP-1 Putty®, Stryker, Kalamazoo, MI). Additionally, *in vitro* studies demonstrate the multi-potency of BMP-2, BMP-6 and BMP-7 in osteogenic and chondrogenic differentiation of marrow-derived mesenchymal stromal cells (MSCs) for tissue engineering and regenerative applications [5–10]. However, the dual nature of BMPs is not well understood and *in vitro* models to study the switch between the osteogenic and chondrogenic lineages have yet to be developed. *In vitro* investigation into the multipotent effects of BMPs relies on specific culture and media conditions to drive undifferentiated cells to the selected lineage, making it difficult to isolate factors that mediate a switch in BMP-induced lineage commitment. Thus, development of a single medium that harnesses the bipotent effect of BMPs to induce both osteogenesis and chondrogenesis could advance the understanding of the role of the cell culture environment in driving cell fate.

Efficient *in vitro* osteogenesis and chondrogenesis depends on culture conditions, and previous studies indicate that several features of the cellular microenvironment are critical for commitment to these lineages. For example, integrin attachment, focal adhesion formation and subsequent cell signaling drives osteogenic differentiation in both monolayer culture and three-dimensional scaffolds [11–13]. Furthermore, enhanced osteogenic differentiation of marrow-derived mesenchymal stromal cells (MSCs) cultured on stiff substrates or at low densities indicate cytoskeletal tension and a spread cell shape may also contribute to early determination of cell fate [14,15]. In contrast, culture conditions maintaining a rounded cell shape such as in alginate and agarose gels, high density micromass culture, and three dimensional pellet culture are favorable for chondrogenesis, suggesting that commitment to the chondrogenic lineage may be dependent on cell shape [16–19]. Furthermore, cell-to-cell interactions and integrin-mediated adhesions to newly formed extracellular matrices may also modulate chondrogenic differentiation [20–23].

In addition to implementing specific culture conditions to induce osteogenesis and chondrogenesis, classical differentiation media for these lineages have distinct formulations. However, unlike culture conditions, media formulations vary greatly and significant overlap can be found between osteogenic and chondrogenic differentiation media. For example, both osteogenic and chondrogenic media may contain BMPs to enhance differentiation

* Corresponding author. Address: Aerospace and Mechanical Engineering, 145 Multidisciplinary Research Building, Notre Dame, IN 46556, USA. Fax: +1 574 631 2144.

E-mail addresses: ckemmis@gmail.com (C.M. Kemmis), avahdati@nd.edu (A. Vahdati), hweiss1@nd.edu (H.E. Weiss), dwagner@nd.edu (D.R. Wagner).

URL: http://www.nd.edu/~dwagner (D.R. Wagner).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.08.135

to either lineage. Additionally, osteogenic media commonly contain serum while chondrogenic media do not, but differentiation to either lineage may be successfully induced in either case.

In light of these facts, we hypothesized that a single medium containing BMPs could induce both osteogenesis and chondrogenesis depending on the cell culture environment. To address this, we investigated the effect of BMP-6 on murine adipose-derived mesenchymal cells (ASCs) in both osteogenic and chondrogenic culture conditions. ASCs are a promising cell source for tissue engineering applications due to their accessibility, abundance and multi-lineage potential; many studies have demonstrated that ASCs have the capacity to differentiate into several tissues, including bone and cartilage [24]. Although the osteogenic effect of BMP-6 on ASCs has not been previously reported, Estes et al. successfully achieved chondrogenic differentiation in ASCs seeded in alginate beads and cultured in medium containing BMP-6 [25]. Furthermore, the dual role of BMP-6 as a factor in both osteogenesis and chondrogenesis has been demonstrated in MSCs using distinct media and culture conditions [6,8].

In this study, we seeded murine ASCs in either monolayer or pellets and induced differentiation with a single medium to investigate the impact of BMP-6 and the microenvironment facilitated by culture conditions on osteochondral differentiation. Here, we provide strong evidence that BMP-6 plays a dual role in differentiation of murine ASCs and demonstrate that the microenvironment found in monolayer and pellet culture may influence the specificity of BMP-6.

2. Methods

ASCs were isolated from male FVB mice at 3–4 weeks of age in accordance with the Notre Dame Animal Care and Use Committee approved protocol as has been described previously [26]. Cells were expanded in growth medium consisting of high glucose DMEM (Mediatech, Manassas, Virginia), 10% heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, California), and 100 IU Penicillin/100 μg/mL Streptomycin (Mediatech). Cells were expanded up to two passages in a humidified incubator at 37 °C and 5% CO_2 before being seeded for experiments.

For monolayer culture, ASCs were seeded at 1300 cells/cm^2 in growth medium and allowed to adhere. Alternatively, ASCs were pelleted by centrifugation at 200,000 cells/well in sterile polypropylene 96-well plates. Differentiation medium was applied to the cells 4 h after seeding, consisting of growth medium supplemented with 100 μg/mL ascorbic acid (Wako, Richmond, Virginia) and 10 mM β-glycerophosphate (Sigma–Aldrich, St. Louis, Missouri) and, when applicable, 100 ng/mL BMP-6 (R&D Systems, Minneapolis, Minnesota) and was replenished every 3–4 days.

To assess proliferation, total DNA content of ASCs seeded in monolayer or pellet was assessed at 0, 3, 7 and 10 days with the Quant-iT PicoGreen dsDNA Reagent Kit (Invitrogen, Carlsbad, California), per manufacturer's instructions. For each timepoint, cell proliferation was expressed as fold change from day 0, $n = 3$.

To evaluate early osteogenic differentiation, alkaline phosphatase activity was quantified in ASCs cultured in monolayer with the colorimetric SensoLyte pNPP Alkaline Phosphatase Assay Kit (Anaspec, San Jose, California) after 7 days using a modified protocol. Alkaline phosphatase activity of the supernatant was measured according to manufacturer's instructions and normalized to total protein concentration via BCA Assay (Pierce, Rockford, Illinois), $n = 4$. Alternatively, cells were fixed and stained with Fast Blue as previously described [26]. ASCs grown in monolayer for 14 days were fixed and stained 0.2% Alizarin red as a late marker of osteogenic differentiation as previously described [26]. Alizarin stain was resuspended with 100 mM hexadecylpiridinium chloride (Sigma–Aldrich) and the absorbance measured at 560 nm using a Victor 3 microplate reader (Perkin Elmer, Waltham, Massachusetts), $n = 6$.

ASCs cultured in pellets were grown for 12 days, at which time sGAG accumulation was quantified. For each sample, eight pellets were pooled, washed with PBS and incubated overnight with 20 μg/mL papain digestion buffer at 58 °C. Samples were briefly vortexed, boiled for 5 min, centrifuged at 12,000*g* for 15 min and the pellet reserved for DNA quantification. The supernatant was collected and proteoglycan content determined via WeisLab sGAG quantitative kit (Alpco, Salem, New Hampshire). The pellet was briefly boiled with 0.02% SDS and incubated overnight with125 μg/mL Proteinase K (Sigma–Aldrich) at 58 °C. DNA was quantified via Quant-iT PicoGreen dsDNA kit and used to normalize sGAG content, $n = 3$. Pellets differentiated for 14 days were washed twice with PBS and fixed for 15 min in 4% PFA and 4% sucrose in PBS. Following fixation, pellets were rinsed in PBS and embedded in optimal cutting temperature compound. 10 μm sections were stained with Alcian Blue Solution and Fast Red Nuclear Stain. The sections were imaged using a Nikon ME600 microscope.

Total RNA was isolated using RNeasy kit (Qiagen, Valencia, California) according to manufacturer's instructions after 3, 7 and 10 days for cells cultured in monolayer or 7, 14, and 21 days for pellet culture, $n = 3$. 1 μg cDNA stocks for each sample were synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). cDNA stocks were analyzed in triplicate (300 ng/well) for gene expression via Fast SYBR Green Mastermix (Applied Biosystems). Data with primers sets specific for *Runx2* (Forward: CGGCCCTCCCTGAACTCT; Reverse: TGCCTGCCTGGGATCTGTA), *Opn* (Forward:AGCAAGAAACTCTTCCA AGCAA; Reverse: *GTGAGATTCGTCAGATTCATCCG*), *Ocn* (Forward: CTGACCTCACAGATCCCAAGC; Reverse: *TGGTCTGATAGCTCGTCACAAG)*, *Agc* (Forward: CTGGGATCTACCGCTGTGAAG; Reverse: *TGTGGAAA-TAGCTCTGTAGTGGA*), *Sox9* (Forward: TTCCTCCTCCGGCATGAGTG; Reverse: CAACTTTGCCAGCTTGCACG), *Col2a1* (Forward: TGGCTGG AGGGTATGACGAG; Reverse: *TTGCCTTGAAATCCTTGAGGG*), were normalized against *18S* (Forward: *AGTCCCTGCCCTTTGTACACA;* Reverse: GATCCGAGGGCCTCACTAAAC). Data were analyzed via $2^{-\Delta\Delta CT}$ method using monolayer or pellet control as a reference for osteogenic and chondrogenic genes, respectively.

Where applicable, statistical significance was determined by one- or two-way ANOVA and Bonferroni's or Tukey's post-test ($p < 0.05$) using GraphPad Prism Software (La Jolla, California).

3. Results

To begin exploration into the roles of culture conditions and BMP-6 during ASC differentiation, we first established the effect of BMP-6 on cell proliferation in both monolayer and pellet cultures, as determined by DNA content (Fig. 1). ASCs seeded in monolayer attached to the culture dish and experienced positive cell growth over the course of 10 days. In contrast, ASCs seeded

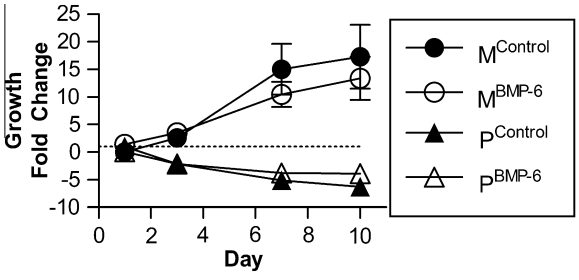

Fig. 1. AMCs were seeded in monolayer or pellets and differentiated with 0 or 100 ng/ml BMP-6. Cell proliferation was determined by assessing DNA content and reported as fold change from day 0, $n = 3$.