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Stimulation of porcine bone marrow stromal cells by hyaluronan, dexamethasone and rhBMP-2

Xuenong Zou[a,b,*], Haisheng Li[a], Li Chen[c], Anette Baatrup[a], Cody Bünger[a], Martin Lind[a]

[a] *Orthopaedic Research Laboratory, Spine Section/Department of Orthopaedics, Center of Nanoscience and Biocompitability, University of Aarhus, Nørrebrogade 44, Building 1A, 8000 Aarhus C, Denmark*

[b] *Department of Orthopaedics, The 5th Affiliated Hospital of Zhongshan (Sun Yat-sen) University, MeiHuaDongLu 52, 519000 Zhuhai, Guangdong, PR China*

[c] *Laboratory For Stem Cell Research, University of Aalborg, Gustav Wieds Vej 10B, 8000 Aarhus C, Denmark*

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Abstract

In the interest of optimizing osteogenesis in in vitro, the present study sought to determine how porcine bone marrow stromal cell (BMSc) would respond to different concentrations of hyaluronan (HY) and its different combinations with dexamethasone (Dex) and recombinant human bone morphogenic protein-2 (rhBMP-2).

Cellular proliferation was determined by ^{3}H-thymidine incorporation into DNA at both Days 2 and 7 when BMSc was cultivated with HY at concentrations of 0, 0.5, 1.0, 2.0 and 4.0 mg/ml. HY accelerated cellular proliferation when compared with cultures in the absence of HY at both Days 2 and 7. BMSc proliferation under the high HY concentration of 4 mg/ml was significantly higher than under the other, lower HY concentrations of 0.5, 1.0 and 2.0 mg/ml.

When BMSc were cultivated under HY at concentrations of 0, 1.0 and 4.0 mg/ml and its 12 combinations with rhBMP-2 at concentrations of 0 and 10 ng/ml and Dex (+, −) at both Days 2 and 7, cellular responses were examined by ^{3}H-thymidine incorporation into DNA, cellular alkaline phosphatase (ALP) activity, and pro-collagen type I C-terminal propeptide production. HY accelerated cellular proliferation irrespective of the presence of Dex and rhBMP-2. HY increased expression of ALP activity at Day 7, whereas had inhibitory effect at Day 2. HY and Dex showed an interaction on expression of ALP acitivity irrespective of the HY dose by Day 7. Collagen synthesis was inhibited by HY irrespective of the presence of other factors at both Days 2 and 7.

When BMSc were cultivated with HY of 4.0 mg/ml alone, its combinations with Dex (+) and 10 ng/ml rhBMP-2, and with DMEM/FBS alone, expression of bone-related marker genes was evaluated by real-time reverse transcription-polymerase chain reaction (Real-time RT-PCR) analysis. Osteocalcin was up-regulated under both rhBMP-2 and HY-Dex-rhBMP-2 at Day 2, as also under 4 mg/ml HY, Dex, HY-Dex, Dex-rhBMP-2, and HY-Dex-rhBMP-2 by Day 7. Type 1α1 collagen was induced by rhBMP-2 on Day 2, and by Dex-rhBMP-2 on Day 7. Osteonectin and type X collagen was only marginally induced by HY at Day 2. Type 1α1 collagen and type X collagen were down-regulated in the presence of 4 mg/ml HY by Day 7.

These results suggest that HY stimulates BMSc proliferation, osteocalcin gene expression, and a secretion of enzymes such as that of ALP activity in vitro. More importantly, HY can interact with Dex and rhBMP-2 to generate direct and specific cellular effects, which could be of major importance in bone tissue engineering.

Keywords: Cell culture; Stem cell; Hyaluronan; Dexamethasone; BMP; Bone tissue engineering

1. Introduction

Hyaluronic acid (HY; also called hyaluronan) is an ancient, highly conservational, extracelluar glycosaminonglycan (GAG) found in all tissues and body fluids of vertebrates as well as in some bacteria. As an almost ubiquitous component of extracellular matrices (ECM) in long bones, HY amounts to 3% of the total GAG [1]. HY and proteoglycans, particularly aggrecan in the osteoid matrix, are involved in mineralization [2]. The distribution of hyaluronan in vitamin D-treated chick bone and the alterations observed in rachitic tissue suggest an essential role for HY in endochondral bone

*Corresponding author. Orthopaedic Research Laboratory, Aarhus University Hospital, Nørrebrogade 44, Building 1A, 8000 Aarhus C, Denmark. Tel.: +45-89494136; fax: +45-89494150.
E-mail address: zxnong@hotmail.com (X. Zou).

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formation [3]. HY interacts with other macromolecules and plays a predominant role in tissue morphogenesis, cell migration, differentiation, and adhesion. The formation and repair of mature hard tissue also requires that mesenchymal precursors divide, differentiate, and migrate to a connective tissue/HY-rich matrices. Locally applied HY with a high molecular weight (190 kDa) has enhanced new bone formation in rat femur wounds [4]. They suggested that HY's stimulation of osteoinduction in this model is due, in part, to its ability to entrap and maintain endogenous BMPs and growth factors liberated from bone margins of the wound. rhBMP-2 in vitro has stimulated the differentiation of osteoblasts from pluripotent mesenchymal cells at only dose concentrations above a specific threshold, while it has been able to induce differentiation of adipocytes at all dose concentrations [5,6]. In both of animal and human studies, rhBMP-2 has proven consistently capable of inducing new bone formation [7–13].

Dexamethasone (Dex) may stimulate both uncommitted stem and committed stromal cells. Uncommitted stem cells from fetal rat calvaria have been observed to differentiate toward an osteogenic lineage when recruited by Dex, presumably by leading to bone cell differentiation at the expense of growth and proliferation [14]. In the porcine BMSc cultures, Dex is sufficient to induce the deposition of mineralized bone matrix and to upregulate bone-related marker genes such as osteocalcin [15,16], type Iα1 collagen [17], and osteonectin [18] in long-term cultures. The osteogenic potential has also been shown with neonatal porcine BMSc. Upon incubation of media containing Dex, porcine BMSc has formed mineralized nodules, which demonstrated ALP-positive cells and a calcified type I collagen-rich matrix [19].

HY binds to cells by direct interaction with cell surface receptors [20] and to extracellular matrix components [21] or to proteins such as hyaladherins [22], each of which produce different biologic functions in specific local environments with varying molecular weights of HY. The cell-signaling function of HY is mediated through CD44, which is abundantly expressed on osteoblasts [23]. Studies using different molecular weights of HY in 1.0 and 2.0 mg/ml dosages to stimulate proliferation and differentiation of mesenchymal stem cell have only recently begun to appear in the literature. Mouse mesenchymal stem cell has showed bone colony formation in vitro with low-molecular weights of HY (30 and 40 kDa) in 1.0 and 2.0 mg/ml concentrations, but they have not shown significant bone colony formation at a high-molecular weight of HY [24]. Low molecular weight HY (60 kDa) has significantly stimulated rat cell growth and osteocalcin mRNA expression in a dose-dependent manner, but it has shown no apparent effects on ALP activity and bone mineralization. Nevertheless, high molecular weight HY (900 and 2300 kDa) has significantly increased ALP activity, osteocalcin mRNA expression and mineralization [25]. Those studies did not assess, however, effects of a higher concentration of HY, nor its combination with Dex and growth factors on the proliferation and differentiation of mesenchymal stem cells. Furthermore, HY (720 kDa) has excised its multiple biologic attributes favourably in early wound healing and tissue regeneration processes [26].

The aim of the present study was to investigate whether different concentrations of HY (800 kDa) can stimulate the proliferation of porcine BMSc and to evaluate the porcine BMSc response to different combinations of HY, Dex and rhBMP-2 in an interest of optimizing osteogenesis for bone tissue engineering.

2. Materials and methods

2.1. Cell culture system of porcine BMSc

Porcine BMSc were isolated using the methods described by Thomson et al. [19]. Briefly, cultures derived from iliac crest bone biopsies of 3-month-old female Landrace pig and thus contained elements of bone marrow stromal cells (progenitor osteoblast cells) and trabecular bone cells (mature osteoblast cells). Iliac crest bone chips were harvested aseptically under general anesthesia. Bone chips with marrow cells were placed into Dulbecco's modified Eagle's medium with Glutamix-1, sodium pyruvate, 4500 mg/l glucose and pyridoxine (*DMEM*, Gibco, BRL) containing 0.1% heparin, washed, and centrifuged at 1200 rpm for 10 min at room temperature. The cells were collected in 75-cm^2 flasks containing 15 ml of DMEM supplemented with penicillin (50 IU/ml; Sigma), streptomycin (50 μg/ml; Sigma), and 10% fetal bovine serum (FBS Australian Origin; Bio Whittaker Europe, Belgium; Lot 8SB0001) and thereby constituted the primary culture. After 24 h, the media were changed in order to remove any non-adherent cells; thereafter, the media were changed 2 times a week. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

The cells of the primary cultures reached confluence after 10–14 days. The cells were then washed twice with PBS buffer, after which 4 ml 0.125% trypsin/5 mM EDTA was added for 5 min at 37°C in order to effect a release. The reaction was stopped by the addition of 6.0 ml of DMEM/10% FBS. After centrifugation at 1200 rpm for 10 min at room temperature, the cells were re-suspended in different media for the development of cultivations.

2.2. Analysis effects of hyaluronan on BMSc proliferation

BMSc's (passage1) were cultivated in 24-well plates at a cell density of 13,157 cells/cm^2 in 1 ml of DMEM/FBS, in both the absence of and supplemented with sodium hyaluronate (M_w = 800 kDa; Lifecore Biomedical, Inc., Chaska, MN). HY with increasing concentrations of 0.5, 1.0, 2.0, and 4.0 mg/ml were used in this study. A triplicate was obtained per cultural medium. Experimental cells were taken from seven individual pigs. The culture media were changed on the 2nd and 5th days. Cellular proliferation was determined by ^{3}H-thymidine incorporation into DNA at time intervals of the 2nd and 7th days.

2.3. BMSc response to hyaluronan, dexamethasone, and rhBMP-2

BMSc response to HY, Dex, and rhBMP-2 (Genetics Institute, Inc., Cambridge, MA) was studied by measuring cell proliferation and osteoblastic differentiation. BMSc's from five different pigs were cultivated in 96-well plates at a cell density of 6000 cells/cm^2 for 2 days and 3000 cells/cm^2 for 7 days. They were then incubated in DMEM/FBS (200 μl) with 12 different combinations (Table 1) of HY (0, 1.0, and 4.0 mg/ml), Dex/Asc/β-GP (+, −), and rhBMP-2 (0, 10 ng/ml). Dex/Asc/β-GP consisted of dexamethasone (1×10^{-8} mol/l, Sigma), ascorbate (82 μg/ml, Merck), and sodium β-glycerophosphate (10 mmol/l, Sigma). A triplicate was obtained per culture medium. The culture media were changed on Day 2 and 5. After 2 and 7 days, cellular proliferation was assayed in terms of ^{3}H-thymidine incorporation into DNA; cellular differentiation was assayed by expression of ALP activity in the cells and PICP production in the media.

Table 1
Twelve different kinds of culture medium (000, 100 … 211) consisting of DMEM/FBS, supplemented with sodium hyaluronate (1.0 and 4.0 mg/ml), dexamethasone (1×10^{-8} mol/l), ascorbate (82 μg/ml), β-glycerophosphate (10 mmol/l), and recombinant human bone morphogenic protein-2 (10 ng/ml)

Medium	DMEM/FBS	HY (mg/ml)	Dex/Asc/β-GP (+,−)	rhBMP-2 (ng/ml)
000	+	0	−	0
100	+	1.0	−	0
200	+	4.0	−	0
010	+	0	+	0
110	+	1.0	+	0
210	+	4.0	+	0
001	+	0	−	10
101	+	1.0	−	10
201	+	4.0	−	10
011	+	0	+	10
111	+	1.0	+	10
211	+	4.0	+	10

DMEM = Dulbecco's modified Eagle's medium; FBS = fetal bovine serum; HY = sodium hyaluronate; Dex = dexamethasone; Asc = ascorbate; β-GP = β-glycerophosphate; rhBMP-2 = recombinant human bone morphogenic protein-2.

DNA synthesis assay was accomplished by a determination of cellular proliferation from ^{3}H-thymidine incorporation into DNA. In all, 25 μCi/ml ^{3}H-thymidine (0.625 μCi/well) (Lifescience, Amersham) was added to each cell layer for the final 20 h of incubation. The incorporation of ^{3}H-thymidine into trichloroacetic acid-perceptible DNA was measured by liquid scintillography (Beta-counter, Wallac, Finland). Intra-assay CV: 9.4%. The result was expressed by cpm value and normalized to cultures incubated in DMEM/FBS alone.

ALP activity was measured in the cell layer after 30 min of incubation with *p*-nitro phenyl phosphate (Sigma) as a substrate at 37°C [27]. Absorbance of p-nitro phenol was determined by micro spectrophotometer at 405 nm. Intra-assay CV: 4.6%. Phenotype expression was estimated by the ratio of ALP in wells supplemented with 10% FBS. The ALP value in this study was adjusted with cell number that cells were counted after methylene blue staining [28] and normalized to cultures incubated in DMEM/FBS alone.

In addition, histochemical staining for ALP was performed randomly in supplementary cultures in order to test the phenotypic stability of the culture system.

Pro-collagen type I C-terminal propeptide (*PICP*) was measured in the conditioned media from 96-well plates of cell culture after incubation for 2 and 7 days. PICP was quantitated in conditioned media by means of a commercial radio-immuno assay, using an antibody that recognizes procollagen C-terminal pro-peptide (PICP [^{125}I], Orion Diagnostica, Finland). ^{125}I radioactivity was counted by using a gamma counter (Wallac, Turku, Finland). Intra-assay CV 9%. The result in this study was adjusted with cell number that cells were counted after methylene blue staining [28] and normalized to cultures incubated in DMEM/FBS alone.

2.4. Expression of bone-related marker genes in response to hyaluronan, dexamethasone, and rhBMP-2

Bone-related marker gene expression of porcine BMSc in response to hyaluronan, dexamethasone and rhBMP-2 was investigated by real-time RT-PCR analysis. BMSc from 3 different pigs were cultivated in 75 cm^2 flask at cell densities of 6000 cells/cm^2 for 2 days and 3000 cells/cm^2 for 7 days in DMEM/FBS alone and secondly in the presence of HY (4.0 mg/ml), Dex/Asc/β-GP (+), rhBMP-2 (10 ng/ml) and their combinations.

Real-time PCR assay was used to quantify osteocalcin, type 1α1 collagen, osteonetin and type X collagen as well as GAPDH-mRNA levels. Total cellular RNA was extracted using Trizol Reagent (Invitrogen, Tastrup, Denmark). RNA samples were DNase I treated and

Table 2
Bone-related marker genes

Gene	Accession number	Oligonucleotides (5′→3′) (up/down)	Product size (bp)
GAPDH	AF017079	GCT TTG CCC CGC GAT CTA ATG TTC GCC AAA TCC GTT CAC TCC GAC CTT	90
Osteocalcin	AW346755	TCA ACC CCG ACT GCG ACG AG TTG GAG CAG CTG GGA TGA TGG	204
Type Iα1 collagen	AF201723	CCA AGA GGA GGG CCA AGA AGA AGG GGG GCA GAC GGG GCA GCA CTC	232
Osteonectin	AW436132	TCC GGA TCT TTC CIT TGC TTT CTA CCT TCA CAT CGT GGC AAG AGT TTG	187
Type X collagen	AF222861	GCC CTT TTG CTG CTG CTA TTG TC GTG TTG GAT GGT GGG CCT TTT ATG	101

used for cDNA synthesis with M-MLV reverse transcriptase (Sigma-Aldrich Denmark A/S) and Random primers (Invitrogen, Tastrup, Denmark). The primers for teat genes were designed using the Primer Select program of the Lasergene software package (DNASTAR, Madison, WI). The respective sequences are listed in Table 2. Prior to routine use, the optimal annealing temperature and predicted size of the PCR product for each gene was verified by gradient tests and electrophoresis. AdvanTaq Plus DNA polymerase (BD Biosciences Clontech) was used to enable a hot-start technique along with the reaction buffer recommended by the manufacturer. To make possible the visualization of PCR products in real time, the SYBR Green I fluorophore (Molecular Probes) was used in a final dilution of 22,000 × from the stock supplied. A two-temperature cycling, consisting of a denaturation step at 95°C for 15 s and annealing/extension step at 60–68°C for 30 s was carried out in an i-Cycler PCR system (Bio-Rad, Hercules, CA). All experiments were performed triplicate for each sample. The relative quantitative expression of bone-related marker genes in each sample was normalized to GAPDH-mRNA level. The fold change of gene expression was normalized to cell culture in DMEM/FBS alone.

2.5. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using multiple analyses of variance (MANOVA) with repeated measures. When significant main effects or an interaction between the main effects was found, specific comparisons were made with paired *t*-tests. Statistical significance was represented by $p<0.05$. Statistical analysis was performed with SPSS version 10.0 statistical software (SPSS, Chicago, IL, USA).

3. Results

3.1. Effect of hyaluronan on BMSc proliferation

HY significantly increased the incorporation of ^{3}H-thymidine into DNA observed at both 2 and 7 days compared to cultures incubated in DMEM/PBS ($p<0.001$). Cellular proliferation in the presence of 4 mg/ml HY was greater than that of 0.5, 1.0 and 2.0 mg/ml HY ($p<0.05$) on Day 2 (Fig. 1A). Cellular proliferation in the presence of 4 mg/ml HY was greater than that of 0.5 and 1.0 mg/ml HY ($p<0.05$) on Day 7 (Fig. 1B). No difference was found among 0.5, 1.0 and 2.0 mg/ml HY.

3.2. Effect of hyaluronan, dexamethasone and rhBMP-2 on cell proliferation

BMSc was cultivated in DMEM/FBS (200 μl) with different combinations of HY, Dex, and rhBMP-2. After 2 days of cultivation, concerned with the within-subject effects on cell proliferation, the effects of HY, rhBMP-2, and Dex-HY, rhBMP-2-HY, Dex-rhBMP-2-HY interactions were significant ($p<0.05$), but the effect of Dex, Dex-rhBMP-2 interaction did not reach significance. Compared to cultures incubated in DMEM/FBS alone, cellular proliferation was significantly increased in the presence of 4.0 mg/ml HY alone and in its combinations with Dex, rhBMP-2, and the latter two together; this was also the case using 1.0 mg/ml HY alone and its combination with Dex ($p<0.05$, Fig. 2A).

After 7 days of cultivation, concerned with the within-subject effects on cell proliferation, the effects of HY, rhBMP-2, and Dex-HY, rhBMP-2-HY interactions were significantly different ($p<0.05$), but the effects of Dex-rhBMP-2 and Dex-rhBMP-2-HY interactions did not reach significance. Compared to cultures incubated in DMEM/FBS alone, cellular proliferation was significantly increased in the presence of 4.0 mg/ml HY alone and in its combinations with Dex or rhBMP-2; this was also the case using 1.0 mg/ml HY alone and its combinations with Dex, rhBMP-2, and the latter two together ($p<0.05$, Fig. 2B).

3.3. Effect of hyaluronan, dexamethasone and rhBMP-2 on ALP activity

Over a time span of 2 and 7 days, ALP activity was significantly increased on Day 7 compared to Day 2 ($p<0.001$, Fig. 3). After 2 days of cultivation, HY

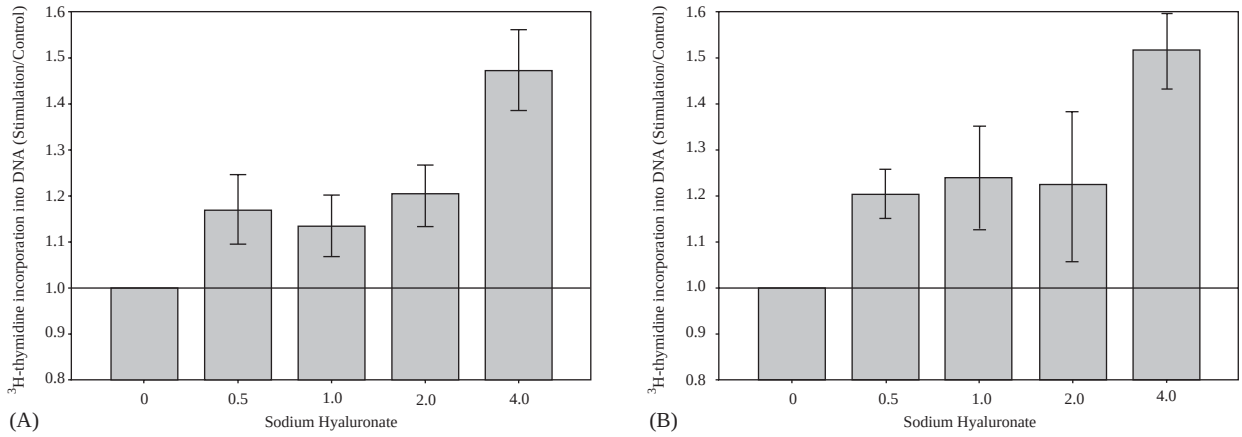

Fig. 1. ^{3}H-thymidine incorporation into DNA at increasing concentrations of 800 kDa sodium hyaluronate (0.5, 1.0, 2.0 and 4.0 mg/ml) on Day 2 (A) and Day 7 (B), normalized to cultures incubated in DMEM/FBS.

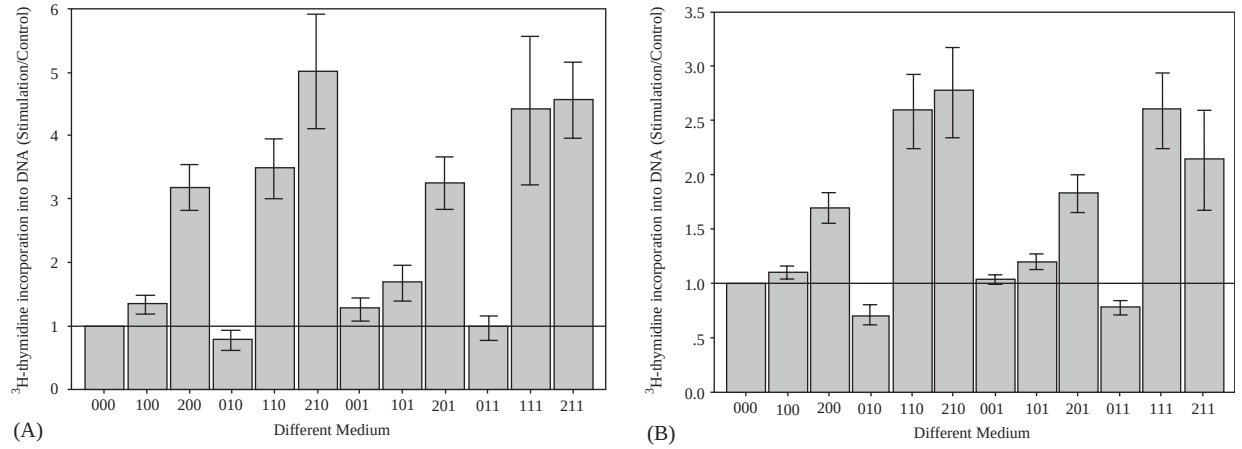

Fig. 2. ^{3}H-thymidine incorporation into DNA (cpm) at cultures incubated in DMEM/FBS with different combinations of HY (0, 1.0 and 4.0 mg/ml), Dex/Asc/β-GP (+, −), rhBMP-2 (0 and 10 ng/ml) on Day 2 (A) and Day 7 (B), normalized to cultures incubated in DMEM/FBS. 000, 100 … 211 are depicted in Table 1.

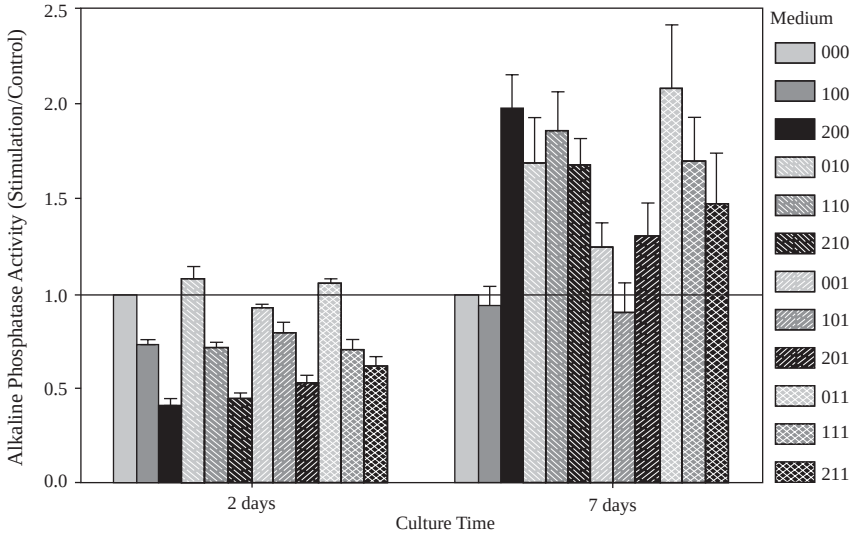

Fig. 3. Alkaline phosphatase activity at cultures incubated in DMEM/FBS with different combinations of HY (0, 1.0 and 4.0 mg/ml), Dex/Asc/β-GP (+, −), rhBMP-2 (0 and 10 ng/ml) on Days 2 and 7. ALP activity were adjusted with cell number and normalized to cultures incubated in DMEM/FBS. 000, 100 … 211 are depicted in Table 1.

significantly decreased ALP activity ($p = 0.005$). In contrast, ALP activity was significantly increased in cells incubated with Dex and rhBMP-2 compared to cultures incubated in DMEM/FBS alone ($p = 0.04$, Fig. 3).

Compared to cultures incubated in DMEM/FBS alone, after 7 days of cultivation, Dex-rhBMP-2 conditioned cultures showed higher ALP activity than did HY-Dex conditioned cultures, when content was increased with cells incubated with Dex alone and Dex-rhBMP-2 ($p = 0.001$). Compared to cultures incubated in DMEM/FBS alone, ALP activity was significantly increased in the presence of Dex and rhBMP-2, 4 mg/ml HY alone and the latter's combinations with Dex; this was also the case using 1 mg/ml HY, Dex and rhBMP-2 together ($p < 0.05$, Fig. 3).

3.4. Effect of hyaluronan, dexamethasone and rhBMP-2 on PICP

After 2 days and 7 day of cultivation, PICP did not show a significant difference on Day 7 when compared to Day 2. HY and interactions with combinations of Dex and rhBMP-2 significantly inhibited collagen type I synthesis ($p < 0.05$). Specifically, compared to cultures incubated in DMEM/FBS alone, PICP were significant lower on Day 2 in the presence of 4 mg/ml HY alone and in its combinations with Dex, rhBMP-2, and the latter two together; this was also the case using 1 mg/ml HY alone and its combinations with Dex and rhBMP-2 ($p < 0.05$, Fig. 4).

Compared to cultures incubated in DMEM/FBS alone, after time intervals of cultivation, on Day 7, PICP was significantly decreased in the presence of 4 mg/ml HY alone and its combinations with Dex, rhBMP-2, and the latter two together; this was also the case using 1 mg/ml HY in combinations with Dex, rhBMP-2, and the latter two together ($p < 0.05$, Fig. 4).

3.5. Effect of hyaluronan, dexamethasone and rhBMP-2 on bone-relate marker gene expression

Gene expression analyses of porcine BMSc cultures in DMEM/FBS alone and in the presence of 4 mg/ml HY, Dex, rhBMP-2 and their combinations were performed for characteristic osteogenic marker genes (Fig. 5). After 2 days of cultivation, osteocalcin induction was increased up to 29-fold in the presence of rhBMP-2 and the combinations of HY-Dex-rhBMP-2 compared with DMEM/FBS alone. By day 7, osteocalcin was up-regulated to 121, 32 and 46-fold when BMSc was cultivated with 4 mg/ml HY, Dex and HY-Dex, respectively. However, induction of osteocalcin gene expression was only 6-fold by rhBMP-2, whereas Dex-rhBMP-2 and HY-Dex-rhBMP-2 increase 22- and 13-fold expression compared with DMEM/FBS alone. Type 1α1 collagen was raised to an 11-fold expression by rhBMP-2 on Day 2, while type 1α1 collagen was caused up to 27-fold induction with combination of Dex and rhBMP-2 on Day 7, when compared with DMEM/FBS alone. Osteonectin and type X collagen was only marginally induced by HY at Day 2. Type 1α1 collagen and type X collagen were down-regulated in the presence of 4 mg/ml HY by Day 7.

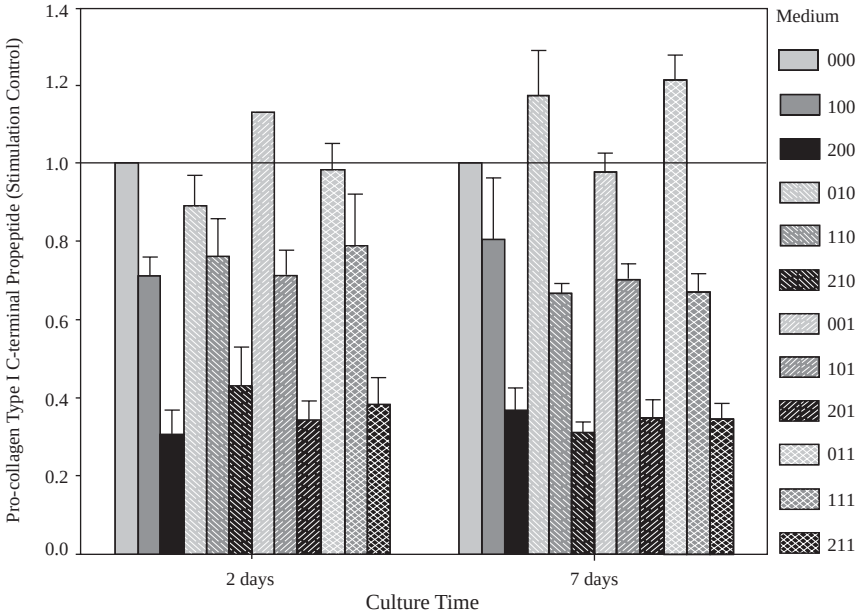

Fig. 4. Pro-collagen type I C-terminal propeptide (PICP) at cultures incubated in DMEM/FBS with different combinations of HY (0, 1.0 and 4.0 mg/ml), Dex/Asc/β-GP (−, +), rhBMP-2 (0 and 10 ng/ml) on Days 2 and 7. PICP was adjusted with cell number and normalized to cultures incubated in DMEM/FBS. 000, 100 … 211 are depicted in Table 1.

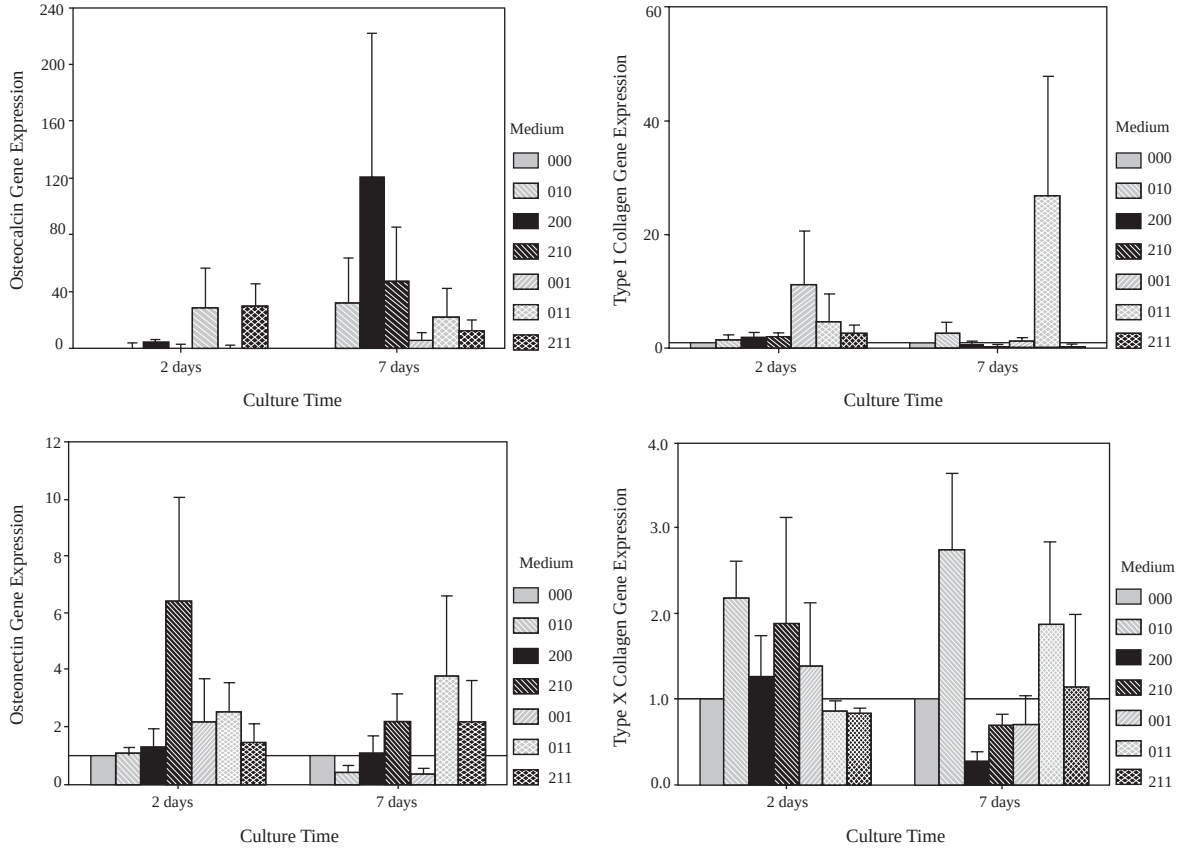

Fig. 5. Relative fold induction of bone-related marker genes in porcine BMSc cultures undergoing osteogenic differentiation. In porcine BMSc cultures with DMEM/FBS alone and the addition of 4.0 mg/ml HY, Dex/Asc/β-GP (+), 10 ng/ml rhBMP-2 or their combinations on Days 2 and 7, the relative quantitative expression of osteocalcin (top left), type Iα1 collagen (top right), osteonectin (bottom left) and type X collagen (bottom right) in each sample was normalized to GAPDH-mRAN level. The relative fold change was normalized to cultures incubated in DMEM/FBS. 000, 100 … 211 are depicted in Table 1.

4. Discussion

Bone has a high potential for self-regenerative repair, with progenitor cells residing in both the periosteum and bone marrow [29]. In bone formation, BMSc in adult bone marrow stroma are believed to play an essential role because they are a major source of osteoprogenitor cells. In vitro, BMSc isolated from bone marrow aspirates or biopsies of rats [30], mice [31], rabbits [32,33], pigs [16,19], and humans [34–37] have been cultivated for study. Purified and expanded mesenchymal stem cells from various species have also been done [38,39]. In those in vitro studies, dexamethasone, ascorbate, and β-glycerophosphate dramatically brought out a demonstration of osteogenic differentiation in long-term cultures with an increase of ALP activity, a deposition of type I collagen, bone nodule formation, and bone-related marker gene expression. Within this complex multilineage cell system of BMSc, studies of environmental determinants of cell proliferation and differentiation may be important for developing an understanding of the full differentiation potential of these cells.

In the present study, we first investigated cellular proliferation of porcine BMSc, a putative source of mesenchymal progenitor cells, in the presence of HY at a low molecule weight of 800 kDa and at the different concentrations of 0.5, 1.0, 2.0 and 4.0 mg/ml. As reported above, the cellular proliferation induced by HY (0.5, 1 and 2 mg/ml) was consistent with the study that used rat calvaria for cell cultures, in which HY at three levels of molecular weight (60, 900 and 2300 kDa) all significantly increased the thymidine uptakes of cells but with dose variations [25]. It is interesting that the distinguishing effect of HY on cellular proliferation in the present study was observed at a high concentration (4 mg/ml), but no clear dose-response difference could be observed at lower doses. This effect of HY was irrespective of the presence of other factors (rhBMP-2 or Dex). The mechanisms of cell proliferation activated by HY tend to increase the volume and surface areas for cell migration and cellular activities and in addition stimulate receptor-mediated events. HY can form a pericellular coat around cells, settle into a cell-poor space in a culture well, and facilitate both cell detachment from its matrix and mitosis in response to

mitogenic factors such as pre-inflammatory mediators and growth factors [40–43]. In the context of an in vitro culture, they can fully express their replicating and dividing potential. A high concentration of HY strands could provide a much larger active surface for surrounding cells, which could bind to the cell surface CD44 receptor, to promote cell migration to a cell-poor space. The high concentration of HY would be offering a hyaluronic acid-rich area that could induce the migratory cells to release more endogenous growth factors and stimulate cell–cell interaction, resulting in faster cell proliferation during early stages.

Extracelluar matrix molecules are involved in both modifying cell responses to growth factors and cytokines and in regulating cell motility, growth, and adhesive interactions. Porcine BMSc responded to HY and their interactions with dexamethasone and rhBMP-2 resulted in a change in their phenotype expression. In the absence of HY, BMSc, once plated, adhere to the bottom of the culture well and initiate the process of condensation, which is a critical step in cellular differentiation. In the presence of HY in either lower or high concentrations, HY settles into cell-poor space and surrounds cells from all sides, which prevents the cells from aggregating in the early stage. HY thus inhibited ALP activity on Day 2 with dose-specific mode irrespective of other factors. As previously mentioned, cells in the process were replicated themselves.

HY binds to cells by direct interaction with adhesion molecule receptors on cell membrane surfaces: such as cluster-determinant CD44 receptor, and receptor for hyaluronate-mediated motility (RHAMM), and ICAM-1 (intercellular adhesion molecule-1) [20,44–46]. They thereby take part in the enhancement of cell growth, differentiation, and functions related to cell adhesion, migration, division as well as enzyme secretion, for example, hyaluronidase.

HY in the present study allowed to continue its processing over a period of time, and by Day 7, a high concentration of HY (4 mg/ml) was used to make more cells to condense. It produced an upregulation of endogenous cellular ALP activity and osteocalcin mRNA expression, which are the mature bone-related markers for osteoblast differentiation. When combined with HY and Dex, an interaction between HY and Dex made the expression on ALP activity and osteocalcin gene earlier than that in another study [25]. HY thus showed a time-specific and concentration-specific mode of action similar to other studies [24,25]. Proteoglycans, such as HY, serve their possible role of being storehouses for growth factors, but they may also interact with such factors to generate direct and specific cellular effects [47]. There may be other important interactions between HY and growth factor in bone repair. As described here, porcine BMSc treated with HY and rhBMP-2 clearly demonstrated cellular proliferation in short-term cultures. Combined with rhBMP-2 and Dex, a high concentration of HY (4 mg/ml) induced early gene expression of osteocalcin on Day 2 and a low concentration of HY (1 mg/ml) increased ALP activity on Day 7. Studies have reported that the extracellular matrix components and cell shape could modulate cellular differentiation and responsiveness to growth factors [48,49]. HY and basic fibroblast growth factor can act synergistically to accelerate new bone formation [50,51].

It has been extensively demonstrated by the present study, that when porcine BMSc are grown in monolayer cultures with HY, the BMSc proliferate and initially induce gene expression of osteocalcin and osteonectin. However, a de-differentiation process took place in which HY reduced pro-collagen type I synthesis and down-regulated the expression of type 1α1 collagen and type X collagen in the early phase of a cell culture over 7 days. This suggests that HY may reduce collagen deposition in the matrix in the early stage, which is similar to the effects of exogenous HY in decreasing wound healing collagen and inhibiting wound scar formation [52–54]. This may occur because BMSc is most likely fibroblastic in appearance in the early proliferation phase and expression of type 1α1 collagen was up-regulated in the differentiation phase after de-proliferation, which detected by Day 24 [16]. It is of interest that the initiation of type X collagen gene expression on Day 2 was reversed by Day 7 in the presence of HY alone and its combination of Dex, in which type X collagen has an important role in the mineralization process during endochondral ossification [55,56]. Because a temporal sequence of events during the process of cellular proliferation has observed in the present study, in which an enhanced expression of alkaline phosphatase occurs immediately after the proliferative period, and later an increased expression of osteocalcin, those genes then would activate the subsequent induction of genes associated with intracellular matrix maturation and mineralization when collagen deposition is promoted. Investigation of collagen deposition and mineralization associated with a prolonged presence of HY is warranted and relevant bone tissue engineering.

A limitation of the present study was the use of an in vitro model that contains a heterogeneous population of cells. However, this well-documented system has been previously used for confirmation of the presence of osteoblast-like cells in cultures through the identification of mineralised bone nodule formation and ALP staining cells [19,57]. The present experimental design did not permit the assessment of mineralised bone nodule formation, because the process was the object of only short-term observation (7 days).

One important property of HY could be that the molecule offers a three-dimensional environment for the

cultivated cells, which would provide the stimulation of metabolism and differentiation through cytoskeletal interactions. Because of its unique physicochemical properties—nonimmunogenicity of the highly purified form—HY has already seen biomedical applications for many years. More recently, the reported benefits of exogenously based biomaterials have been shown for tissue repair purposes. Already in this field, products employ either pure HY [58], or derivatives of HY, which use cross-linking [59], esterification [60–62] or other chemical modification techniques to improve their physical handling and stability characteristics. HY's biological function on BMSc could be more important in the interest of bone tissue engineering.

To summarize in conclusion, this study demonstrates that HY accelerates cellular proliferation, increases ALP activity and osteocalcin gene expression, and inhibits pro-collagen type I synthesis and the expression of type 1α1 collagen when porcine BMSc is cultivated with HY alone or combined with Dex or rhBMP-2 or the later 2 together. ALP activity and the expression of type 1α1 collagen was also increased in the presence of Dex and rhBMP-2. More importantly, HY interacted with Dex and rhBMP-2 to generate direct and specific cellular effect, which would be of major importance in bone tissue engineering.

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