THE EFFECT OF HYALURONAN ON OSTEOGENIC DIFFERENTIATION OF PORCINE BONE MARROW STROMAL CELLS IN VITRO

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INTRODUCTION
Hyaluronan (HA) plays a predominant role in tissue morphogenesis, cell migration, proliferation, and cell differentiation. Previous results from the author’s group demonstrated that medium size HA (800 KDa) can stimulate porcine osteoblast proliferation, osteocalcin gene expression and increase ALP activity in vitro in a dose-dependent manner (from 1.0 mg/ml to 4.0 mg/ml) at the early stage (7-day culture). However, the effect of HA on the whole osteogenic differentiation progress has not been known. In vivo, HA gel mixed with bone marrow stromal cells (BMSC) can replace autograft as an osteoinductive agent when loaded into a porous tantalum ring in the porcine anterior lumbar interbody fusion (ALIF) model. The aims of the present study were to investigate whether (i) the proliferation presence (over a three-week culture period) of high concentration (4.0 mg/ml) 800 KDa HA, and (ii) pre-cultivation of pBMSC with HA to modify the expression of differentiation-related genes in pBMSC. In particular, we studied the role of HA combined with dex, β-GP and Asc on osteogenic differentiation of pBMSC.

METHODS

1. Cell isolation
Bone marrow (about 30 ml) was obtained from 3-month-old female Danish landrace pigs by means of aspiration from the medullary cavity of the proximal tibia using a bone marrow aspiration needle. The nucleated cells were collected by means of Ficoll density gradient centrifugation and finally resuspended in basal medium. Basal medium consisted of Dulbecco’s modified Eagle’s medium with Glutamix-1, sodium pyruvate, 4500 mg/l glucose and pyridoxine (DMEM, Gibco, BRL) supplemented with 10% FBS and 1% PS. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. After 72 hours, the medium was changed in order to remove any non-adherent cells. The first passage of cells was used for experimental manipulations.

2. Cell expansion
After confluence, the cells were detached using 0.125% trypsin/5 mM EDTA, plated at 6000 cells/cm² in either basal medium or basal medium supplemented with 4.0 mg/ml 800 KDa HA and cultured for 7 days. Cultures from some plates were collected for gene expression. Additionally, BMSC from previous expansion in basal medium including HA were subcultivated in basal medium, osteogenic medium (Basal medium + 10 mM dex +10 mM β-GP+82 g/ml ascobic acid), HA (Basal medium + 4.0 mg/ml HA) and osteogenic medium plus HA (Osteogenic medium + 4.0 mg/ml HA), respectively. Meanwhile, BMSC from previous expansion in basal medium were subcultivated in basal medium and osteogenic medium.. Cultures were harvested at day 7, day 14 and day 21 for calcium deposit assay and gene expression analysis.

Cell number was quantified by SYBR green I assay. gene expression was analyzed by means of real-time PCR.

3. Statistical analysis
Data were expressed as mean ± standard deviation (SD). Statistics were assessed using SPSS 10.0 (Chicago, IL). Cell proliferation and calcium deposit data were analyzed by one-way ANOVA. The data of gene expression were determined using two-way ANOVA (time x treatment) followed by paired t-test. P value <0.05 was considered significant.

RESULTS

1. Effect of HA on cell proliferation
HA alone or mixed with dex, β-GP/Asc significantly increased cell number (P<0.05). There was no additive effect between them. Dex, β-GP/Asc or their combination showed no significant difference on cell proliferation (P>0.05).

2. Effect of HA on mineralization
In basal medium or mixed with HA alone, calcium deposit was not detected. Mineralization was increased in osteogenic medium. The addition of HA in osteogenic medium increased calcium deposit, P=0.05.

3. Effect of HA on hyaluronan synthase 2 (has2) mRNA expression

We also investigated whether HA induces has2 gene expression. The addition of exogenous HA increased has2 gene expression level by BMSC in comparison with the cultures in basal medium during 7-day pretreatment of HA.

4. Effect of HA on basal level of differentiation related genes in pBMSC
Interestingly, HA decreased basal level of cbfa1, osterix, ALP, Col1, osteonectin (ON) gene expression, whereas it slightly increased basal level of sox9 during 7-day pretreatment of HA, p<0.05.

5. Effect of HA on bone-related genes in pBMSC during 21-day osteogenic differentiation
On day 7, cbfa1, ALP and Col1 genes were increased when pBMSC were cultivated in osteogenic medium, whereas they were decreased when pBMSC were cultivated in HA alone. On day 14, HA mixed with osteogenic medium increased cbfa1 and ALP expression (1.3 fold and 1.9 fold respectively) and osteogenic medium plus HA had no effect on osteogenic signal pathway. In addition, HA may play a role as reservoir of hyaluronans to induce osteogenic differentiation. In an osteogenic inducing condition, HA may stimulate porcine osteoblast proliferation, osteocalcin gene expression were determined using two-way ANOVA (time x treatment) followed by paired t-test; P value <0.05 was considered significant.

Fig 1. Real-time RT-PCR shows relative expression of cbfa1 (A), ALP (B), Col1 (C), OC (D) in pBMSC cultures with medium depicted in the materials and methods on day 7, day 14 and day 21.

DISCUSSION
The mechanism of cell proliferation by HA may be explained by the signal events stimulated by the interaction between HA and its receptor CD44 expressed on BMSC. CD44 is a principal receptor for HA, and the interaction between them has been implicated in multiple cellular functions, including cell proliferation and differentiation. Moreover, higher levels of endogenously synthesized HA were reflected in a higher cell proliferation rate.

The addition of dex, β-GP/Asc can inhibit the increase of sox9 (data not shown) induced by HA and induce osteogenic differentiation. HA in combination with dex, β-GP/Asc decreased cbfa1 and ALP gene expression compared with the dex, β-GP/Asc alone during proliferation on day 7. Following a period of time, BMSCs cease proliferation and accelerate differentiation. HA mixed with dex can synergistically induce osteogenic differentiation. In an osteogenic inducing condition, HA may stop the intracellular chondrogenic signal pathway and open the osteogenic signal pathway. In addition, HA may play a role as reservoir for growth factor within the FBS and dex due to its physicochemical (negative charges) properties and increases cbfa1 and ALP gene expression. Whether the synergetic role of HA resulted from HA itself or from the local concentration of its breakdown products remains to be elucidated. Hyaluronidase 2 (HAAL 2) is generally thought to degrade HA to a specific 20 kDa, which is involved in mineralisation to promote bone formation. We have not analysed porcine HAAL gene expression due to the lack of its sequence. BMSCs exposed to treatment with HA were consistent to the study that fibroblasts exposed to low concentration of HA stimulated collagen synthesis while high concentration had no change. In conclusion, these in vitro data demonstrate that HA has an important role in both the early and the later stages of bone formation. It can increase proliferation and osteogenic differentiation.

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