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Extensive CD44-dependent hyaluronan coats on human bone marrow-derived mesenchymal stem cells produced by hyaluronan synthases HAS1, HAS2 and HAS3

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ABSTRACT

Hyaluronan (HA), a natural extracellular matrix component, has been considered as an important constituent of the stem cell niche, and successfully used as 3D scaffolds for the chondrogenic differentiation of stem cells. However, the expression levels of HA synthases (HAS1, 2 and 3) and the synthesis of HA by stem cells have remained unknown, and were studied here in the human bone marrow-derived mesenchymal stem cells (hMSCs). Nine hMSCs from different donors were cultured as monolayers with MSC culture medium supplemented with FGF-2. The amount of HA secreted into medium was studied by an ELISA-type assay, and HA bound to cell surface by live cell microscopy. The expression of HASs was analyzed by real time RT-PCR and immunostainings. The HA receptor CD44 was studied by immunocytochemistry. An intense HA coat surrounded the plasma membrane and its protrusions in all nine hMSCs. Displacement assay with HA oligosaccharides indicated that HA coat was at least partly dependent on CD44, which showed similar, relatively high expression in all hMSCs. All HAS isoenzymes were detected, HAS1 showing the largest and HAS3 the smallest range of expression levels between the hMSCs. The secretion of HA ranged between 22.5 and 397.4 ng/10,000 cells/24 h, and could not be clearly assigned to the mRNA level of a certain HAS, or a combination of the isoenzymes. This suggests that post-transcriptional and post-translational factors were involved in the adjustment of the HA secretion. In conclusion, all hMSCs expressed high levels of HAS1–3, secrete large amounts of HA, and surround themselves with a thick HA coat bound to CD44. The results suggest that hMSC has the potential for autocrine maintenance of the HA niche, important for their stemness.

1. Introduction

Mesenchymal stem cells (MSCs), isolated from bone marrow, have been extensively investigated (Friedenstein et al., 1970; Caplan, 1991; Prockop, 1997), and demonstrate a good potential for the cartilage repair and regeneration (Barry, 2003; Quintavalla et al., 2002; Radice et al., 2000; Yang et al., 2012). They can be expanded in reasonable quantities without a loss in their ability to differentiate (Barry, 2003; Kaitainen et al., 2013; Pittenger et al., 1999; Quintavalla et al., 2002). In three-dimensional cultures or gel-like matrix supports they mature in the presence of defined chondrogenic medium into chondrocytes, which produce type II collagen and cartilage proteoglycan (Nöth et al., 2002; Pittenger et al., 1999; Puetzer et al., 2010; Quintavalla et al., 2002; Radice et al., 2000).

Hyaluronan (HA), a nonsulfated highly hydrated glycosaminoglycan, is a natural extracellular matrix (ECM) component of the cartilage tissue. It allows cell adhesion and regulates cell proliferation, mobility and angiogenesis via activation of its receptors, CD44 (Ishida et al., 1997; Sironen et al., 2011; Slevin et al., 2002) and RHAMM (Gao et al., 2008; Turley et al., 1991). Hyaluronan is synthesized at the inner face of the cell plasma membrane by the integral membrane proteins called hyaluronan synthases (HASs), which use UDP-N-acetyl glucosamine and UDP-glucuronic acid to alternately add N-acetyl glucosamine and glucuronic acid to form HA (Prehm, 1984; Itano et al., 1999, Qu et al., 2007). The newly formed HA molecules are simultaneously translocated into extracellular space

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and often remain attached to cell surface, forming a relatively thick coat (Rilla et al., 2008). Three isoforms of HAS (HAS 1, 2 and 3) have been identified in various cell types (Adamia et al., 2005; Calabro et al., 2002; Itano et al., 1999; Recklies et al., 2001; Tammi et al., 2011). Hyaluronan and HASs have been indicated to have a major impact on normal, diseased and malignant cell behavior (Adamia et al., 2005; Golshani et al., 2008; Hiscock et al., 2000; Nykopp et al., 2009; Wang et al., 2006), and also stem cells (Calabro et al., 2002; Ducale et al., 2005; Zhu et al., 2006).

Hyaluronan has been considered as a potential niche matrix for stem cells due its function as a regulator of cellular behavior during embryogenesis, morphogenesis, migration, proliferation, differentiation and drug resistance (Chen et al., 2007; Jha et al., 2011; Liu et al., 2008; Liu et al., 2009; Solis et al., 2012). It has been shown in human chondrosarcoma-derived chondrocyte-like cell line (HCS2/8) that CD44 regulates cell proliferation and matrix synthesis in cartilage microenvironment by a variety of stimulatory signals through its adhesion to HA (Ishida et al., 1997). The chondrocyte pericellular matrix in the cartilage is directly assembled and retained at the cell surface through HA and its receptors (Knudson, 1993). Thus, HA-based hydrogel potentially provides a niche for MSCs in cartilage tissue engineering based on its abundance, natural pre-chondrogenic properties and easy tenability (Chung and Burdick, 2009; Erickson et al., 2009; Loken et al., 2008; Matsiko et al., 2012). On the other hand, accumulation of HA has been shown in injured cartilage (Lammi et al., 2001), and can potentially attract exogenous MSCs to the wounded area, similarly to CD44-mediated localization of MSCs into injured kidney (Herrera et al., 2007). Hyaluronan attached to the surface of connective tissue progenitor has been used for the selection of these cells from bone marrow aspirates for subsequent osteogenic induction (Caralla et al., 2012; Caralla et al., 2013).

As discussed above, there are several indications for the importance of HA in the mesenchymal stem cell niche, reported in numerous papers during recent years (Kim et al., 2012; Liu et al., 2008; Liu et al., 2009; Pilarski et al., 1999; Solis et al., 2012). However, as far as we know there are no publications that have measured HA production by the actual mesenchymal stem cells. The present study shows that human bone marrow mesenchymal stem cells express high levels of all three HAS enzymes, synthesize large amounts of hyaluronan, and surround themselves with an extensive HA coat attached to their CD44 receptor. The data suggest that the HA-rich stem cell niche is at least partly an autocrine function of the bone marrow stem cells.

2. Materials and methods

2.1. Materials

Alpha-Modified Eagle's Medium (α-MEM), L-glutamine, fungizone and penicillin/streptomycin were obtained from Euroclone (Pero, Italy) and fetal bovine serum (FBS) from Hyclone (Thermo Scientific, Cramlington, UK). Fibroblast growth factor-2 (FGF-2) and transforming growth factor-β_3 (TGF-β_3) were from Peprotech (London, UK). Toluidine blue was purchased from Serva (Heidelberg, Germany). The antibody against Stro-1 was from R&D Systems (Minneapolis, MN, USA), monoclonal antibodies against CD44 (H4C4 and Hermes 1), CD45 and CD105 from Developmental Studies Hybridoma Bank (Iowa City, IA, USA), and anti-CD90 from Millipore hMSC characterization kit (Billerica, MA, USA). Santa Cruz Biotechnology (Santa Cruz, CA) provided the antibodies for HAS1 (2 μg/ml, sc-34021), HAS2 (2 μg/ml, sc-34067) and HAS3 (2 μg/ml sc-34204). The fluorescein isothiocyanate (FITC)-labeled secondary goat anti-mouse antibody was from Chemicon (Temecula, CA, USA), and Texas Red-labeled anti-goat antibody from Vector Laboratories (Burlingame, CA, USA). Non-immune mouse IgG was from Sigma. For immunohistochemical stainings, Envision+ System-HRP kit (Dako, Glostrup, Denmark) was used for detection. All the other chemicals were from Sigma–Aldrich (St. Louis, MO, USA) or Fluka (Buchs, Switzerland).

Several formulae of media were used for the specific purposes. Mesenchymal stem cell culture medium consisted of α-MEM supplemented with 10% FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine and 10 ng/ml of FGF-2 (freshly added). Chondrogenic medium was serum-free α-MEM (Hyclone, South Logan, UT, USA) supplemented with 10 ng/ml TGF-β_3, 50 μg/ml 2-phosphate-L-ascorbic acid trisodium salt, 40 μg/ml L-proline hydrochloride solution, 100 μg/ml pyruvate, 1% ITS + 3 liquid media supplement and 100 nM dexamethasone. Osteogenic medium contained α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 nM dexamethasone, 20 mM β-glycerophosphate (Fluka), and 50 μg/ml 2-phosphate-L-ascorbic acid trisodium salt. Adipogenic medium was made of α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 50 μM indomethacin.

2.2. Isolation and cultivation of human bone marrow-derived mesenchymal stem cells

Human MSCs were isolated from bone marrow materials taking advantage of their property to adhere onto plastic surfaces as described in the previous study (Kaitainen et al., 2013). Samples from nine donors were examined separately. Human bone marrow material samples were collected from surgical specimens at the local university hospital during hip joint replacement operations (North-Savo Health Care District Ethical Committee license no. 62//2010). The hMSCs were cultivated with MSC culture medium for approximately 2 weeks until 90% confluency before freezing for storage.

Passage 2 hMSCs or human chondrosarcoma (HCS2/8) cells were thawed and expanded in 10 cm plate in the previously described MSC culture medium until 90% confluency was reached. After harvesting with trypLE and washing with PBS, the cells were seeded into 6-cm-culture plate, 24-well-plate or μ-chamber slides (Ibidi, Martinsried, Germany) with MSC culture medium. When the seeded cells (0.02×10^6) in μ-chamber slides reached 60% confluency, the cells were either directly stained with fluorescent HA binding complex (fHABC) for HA coat (Rilla et al., 2008), or fixed with 4% paraformaldehyde for immunocytochemical assays. For real-time quantitative RT-PCR analyses, the cells at the original density of 0.3×10^6 cells per 6-cm-plate were cultured in 5 ml of MSC culture medium until they reached 90% confluency. The cellular RNA was dissolved with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and stored at −70 °C until further analyses.

2.3. Chondrogenic, osteogenic and adipogenic differentiation of hMSCs

The chondrogenic differentiation capacity of each batch of hMSCs was evaluated after expansion in monolayer cultures. Passage 3 hMSCs at cell density of 0.3×10^6 per 10 cm cell culture dish were cultured in MSC culture medium until 95% confluency. After the cells were harvested with trypLE and PBS washings, the chondrogenic differentiation was performed with 0.5×10^6 of hMSCs in pellet cultures in chondrogenic medium for 4 weeks as described previously (Kaitainen et al., 2013; Qu et al., 2013). Pellets grown in regular MSC culture medium in the absence of chondrogenic factors was used as a non-differentiated control. At the end of the cultures, the fixed pellets were evaluated with immunocytochemical staining of toluidine blue for proteoglycans, and immunostaining