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The effects of covalently immobilized hyaluronic acid substrates on the adhesion, expansion, and differentiation of embryonic stem cells for *in vitro* tissue engineering

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ABSTRACT

We investigated the *in vitro* effects of the molecular weight (MW) of hyaluronic acid (HA) on the maintenance of the pluripotency and proliferation of murine embryonic stem (ES) cells. High (1000 kDa) or low (4–8 kDa) MW HA was derivatized using an ultraviolet-reactive compound, 4-azidoaniline, and the derivative was immobilized onto cell culture cover slips. Murine ES cells were cultured on these HA surfaces for 5 days. High-MW HA interacted with murine ES cells via CD44, whereas low-MW HA interacted with these cells mostly via CD168. ES cells grown on both high- and low-MW HA appeared undifferentiated after 3 days. However, more cells adhered, proliferated, and exhibited greater amounts of phospho-p42/44 mitogen-activated-protein-kinase on low- compared with high-MW HA. Expression of Oct-3/4 and phosphorylation of STAT3 were enhanced by ES cells on low-MW HA, not on high-MW HA. After release from HA, cells cultured on low-MW HA in the presence of differentiating medium showed enhanced expression of α-SMA or CD31 compared with cells cultured on high-MW HA. It was concluded that low-MW HA substrates were effective in maintaining murine ES cells in a viable and undifferentiated state, which favors their use in the propagation of ES cells for tissue engineering.

1. Introduction

Embryonic stem (ES) cells are used as a source of cells for tissue engineering scaffolds and exhibit the ability to proliferate in an undifferentiated state, yet they maintain pluripotency and differentiate into many specialized cell types when induced by growth factors and appropriate chemical stimuli [1]. However, the controlled renewal of ES cells remains a challenge [2,3]. Routine protocols for the propagation of ES cells require mouse fibroblast or human feeder layers for cell growth, subsequent enzymatic cell removal, and spontaneous differentiation in cultures of embryoid bodies. Each of these steps involves significant variability of culture conditions; therefore, methods that will simplify and provide a better control of these processes are needed. Nagaoka et al. maintained murine ES cells in an undifferentiated state in culture in E-cadherin-coated plates, without colony formation [4]. Efforts have been made to culture human ES cells (hESCs) without feeder layers by altering oxygen tension [5], adding growth factors [6], or culturing cells as floating clusters [7]. However, there remains a need for a more simplified culture system that does not require a feeder layer, maintains ES cells in a pluripotent, yet undifferentiated, state, and allows their proliferation and induced differentiation after release from culture.

Hyaluronic acid (HA), which is a nonsulfated linear polysaccharide of (1-β-4) D-glucuronic acid and (1-β-3) *N*-acetyl-D-glucosamine, correlates with gene expression, signaling, proliferation, motility, adhesion, metastasis, and morphogenesis of hESCs *in vivo* [8]. In humans, the HA content *in vivo* is greatest in undifferentiated ES cells and during early embryogenesis [9]. Therefore, HA is being used widely to culture and propagate stem cells [2,10,11], and the fabrication of biodegradable 3D HA scaffolds enriched with multipotent stem cells seems to be a promising strategy for the repair of irreversibly injured tissues. HA scaffolds support undifferentiated proliferation of hESCs in the absence of feeder layers and retain their ability to differentiate after release from HA hydrogels [2]. HA substrates also protect hematopoietic stem cells from the cytotoxic effects of 5-fluorouracil, by serving as a protective niche for these cells [12].

However, it is known that the effect of HA on the cellular environment varies according to its molecular weight (MW). For example, it is generally accepted that low-MW HA (LMW HA) is angiogenic and is involved in tumor metastasis, whereas high-MW HA (HMW HA) is viscous, non-cell adhering, and lubricating. HMW HA is believed to adhere to cells in a polyvalent manner leading to

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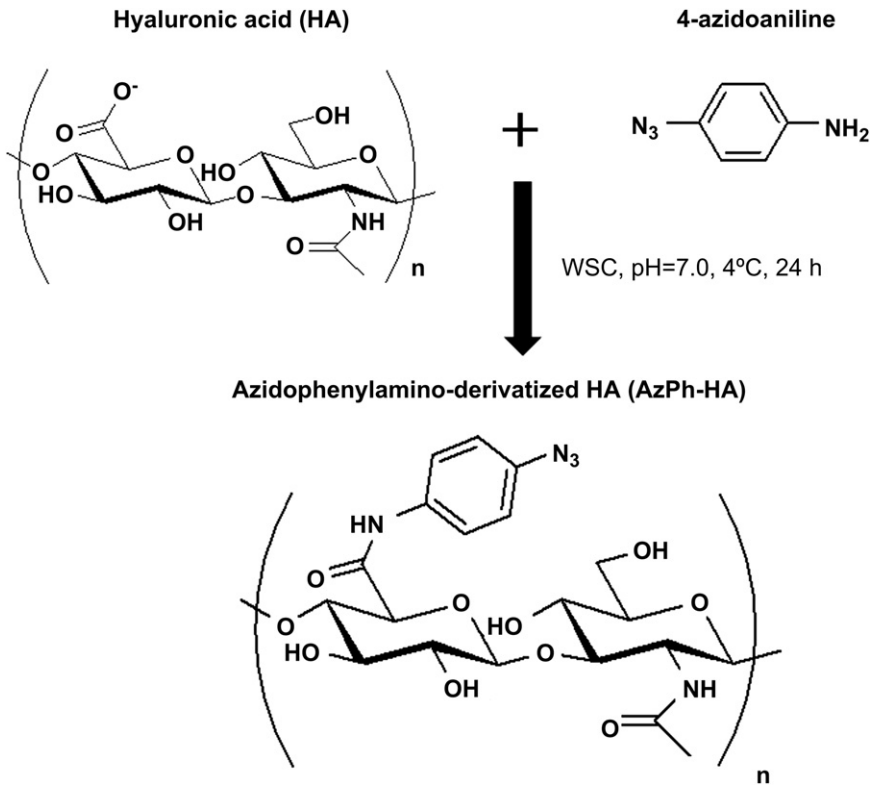

Fig. 1. Scheme used for the chemical synthesis of AzPh-HA. HA consists of repeated units of the monomer sequence comprising D-glucuronic acid and N-acetyl-D-glucosamine. Each single monomer sequence is referred to as "n".

the formation of pericellular sheaths that do not facilitate cell–cell and cell-growth factor interactions. In contrast, LMW HA interacts with cellular receptors in a monovalent manner and may lead to clustering of cell-surface receptors (e.g., CD44) to activate intracellular signaling cascades [13].

We have demonstrated previously the role of very-LMW HA (HA oligomers) in regenerating vascular elastin, thereby establishing HA oligomers as suitable candidates for the fabrication of regenerative vascular scaffolds [14,15]. The molecular-size-specific effects of HA have also been demonstrated in other cell types, such as chondrocytes [16], osteoarthritic cells [17], cancer cells [18–20], endothelial cells [21], and in breast and ovarian tumor cells [20]. Based on these responses of different cell types to variations in the MW of HA, it is reasonable to estimate differences in ES cell behavior in response to HA of varying MW.

Our group has immobilized biomolecules onto 2D substrates for tissue engineering using a technique known commonly as "photoimmobilization" [22,23]. This technique offers the unique versatility of immobilizing various kinds of biomaterials onto culture substrates [24–27] and poses itself as a "high-throughput screening system" for evaluating the properties of any biomaterial regarding cells cultured on such surfaces. In addition, this method uses smaller amounts of material for immobilization compared with soluble boluses, which need to be supplemented repeatedly to cell cultures. Prior studies include the photoimmobilization of HA on PET films [28]. Using similar techniques, we chose to photoimmobilize HMW and LMW HA onto tissue culture substrates and to compare and contrast their effects on murine ES cells *in vitro*. As we hypothesized the presence of differences in the effect of HA on ES cell behavior according to culture on either HMW or LMW HA, we attempted to explore the presence and interaction of the HA receptors CD44 and CD168 on these ES cells and examined how these cell receptor–HA interactions determined cell fates *in vitro*.

2. Materials and methods

2.1. Synthesis and characterization of azidophenyl-derivatized HA

Using 4-azidoaniline hydrochloride, 1-ethyl-3-[3-(dimethylamino) propyl]-carbodiimide hydrochloride (water-soluble carbodiimide, WSC; Wako, Osaka, Japan), HMW HA (~1000 kDa; Wako), and LMW HA (4–8 kDa; R&D Systems Inc., Minneapolis, MN, USA), azidophenyl-derivatized HA (AzPh-HA) was prepared according to the scheme outlined in Fig. 1. Briefly, HA (18 mg), 4-azidoaniline (7.75 mg), and WSC (10 mg) were dissolved in 5 mL of 0.3 M NaH_2PO_4 at pH 7.0 and the solution was stirred at 4 °C for 24 h. Then, the solution was ultrafiltrated (filtration below a MW of ~10,000; Millipore MoleCut II, Millipore, Billerica, MA, USA) and the residue was washed repeatedly with distilled water until the absence of 4-azidoaniline in the filtrate was confirmed by ultraviolet absorption at 257 nm. The purified solution was then lyophilized to yield photoreactive LMW HA, HMW HA, or AzPh-HA. The amount of azidophenyl groups incorporated was calculated as reported previously [29].

2.2. Photoimmobilization of HA

The scheme used to create a photoimmobilized AzPh-HA micropatterned substrate is outlined in Fig. 2. An aqueous solution of AzPh-HA (200 μL of a 0.2% w/v solution) was pipetted onto a cover slip and air-dried at room temperature under a sterile laminar-flow hood. Subsequently, the cover slip was irradiated with an ultraviolet lamp (100 W, Koala, Ted Pella Inc., Redding, CA, USA) at a distance of 5 cm for 10 s in the presence or absence of a photomask (Nippon Filcon Co. Ltd., Osaka, Japan). The cover slip was then washed with distilled water until the absence of AzPh-HA in the wash was confirmed by UV spectroscopy. The immobilized AzPh-HA pattern retained on the cover slip was observed using a bright-field microscope (Olympus Co., Tokyo, Japan) and a fluorescence microscope (Rhodamine filter, 590 nm; Olympus). These AzPh-HA immobilized cover slips were washed in sterile PBS, UV irradiated for 10 min, and used for cell culture.

2.3. Cell culture

Thermanox plastic cover slips (Nalge Nunc International, Rochester, NY, USA) with a diameter of 5 mm were coated using either HA or gelatin (0.2% w/v, Millipore), to serve as controls. All cover slips were inserted into 12-well culture plates (Nalge Nunc International). Feeder-free murine ES cells and a germ-line-competent murine ES cell line were used in all cell culture studies [30] and